

VITAMIN D, INFECTION, AND INFLAMMATION IN PREGNANT
ADOLESCENTS

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Vitamin D is thought to modulate both innate and adaptive immune responses at the cellular level, but the relationship between vitamin D, infection, and inflammatory processes across pregnancy is unclear.

The first objective of this study was to describe the prevalence of clinically diagnosed infections and to identify risk factors for infections. The second objective was to examine associations between vitamin D status, systemic inflammatory biomarkers, and infections across pregnancy. The third objective was to evaluate associations between vitamin D and placental antimicrobial peptide expression.

To address these study aims, the prevalence of clinically-diagnosed maternal infections and placental inflammation was determined and potential risk factors were identified. We determined that African-American race, higher pre-pregnancy body-mass-index (BMI), younger age at diagnosis, and low intake of fat-soluble vitamins A and D were associated with greater infection prevalence. Using archived serum samples, we found positive associations between serum 1,25-dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}$) and the pro-inflammatory cytokines IL-6 and TNF- α during pregnancy, but inverse associations were observed between $1,25(\text{OH})_2\text{D}$ and both pro-

inflammatory cytokines at delivery. In addition, lower serum 25-hydroxyvitamin D 25(OH)D status was associated with positive diagnosis of candida and bacterial vaginosis infections in these pregnant adolescents. In archived placental tissue samples, we found that the placental expression of vitamin D proteins (cubilin and CYP27B1) mediated the relationship between placental antimicrobial peptide expression and positive diagnosis of recto-vaginal group B streptococcus colonization in the mother. In summary, our results suggest that pregnant African American adolescents are at an increased risk for having sexually-transmitted and other vaginal infections during pregnancy, lower vitamin D status may increase risk of vaginal infections during pregnancy, and that vitamin D metabolites are associated with biomarkers of systemic inflammation and with the placental expression of antimicrobial peptides. Future research is needed to evaluate the potential for vitamin D supplementation to prevent and/or treat urogenital infections across gestation.

BIOGRAPHICAL SKETCH

Christine Akoh was born in College Station, Texas, but she spent most of her childhood growing up in Athens, Georgia. Both of her parents are Nigerian immigrants, and they instilled the importance of education for Christine and her three siblings at an early age. At the age of 16, Christine obtained her first job as a student researcher through the University of Georgia (UGA) College of Agriculture and Environmental Sciences' Young Scholars Program (YSP). For three summers (2006, 2007, and 2008), she conducted research projects in the pharmacology, entomology, and food science departments, respectively. Christine graduated from Oconee County High School in 2008 and from UGA in 2012 with a BSA in Food Science. While a student at UGA, Christine conducted research for two years through the Center for Undergraduate Research Opportunities (CURO) Apprentice Program and the CURO Summer Fellowship in the fields of infectious disease immunology and food microbiology. Outside of research, Christine was involved in various professional societies at the national and local level: Institute of Food Technologists, Minorities in Agriculture, Natural Resources and Related Sciences, and International Association of Students in Agricultural and Related Sciences. She also participated in Bollywood fusion and breakdance teams during her undergraduate years.

After graduating from UGA, Christine began her PhD program in Human Nutrition at Cornell University with a focus on maternal and fetal health under the mentorship of Dr. Kimberly O'Brien. While at Cornell, Christine continued her passion for dance and performed with Sabor Latino Dance Ensemble for 3 years. In

the future, Christine plans to seek a short-term postdoctoral position and later pursue a health professions degree.

This dissertation is dedicated to my parents Casimir and Celine Akoh. I am thankful for their everlasting support and faith in my academic pursuits and for being great role models. Furthermore, I would like to thank God for always guiding me along the way and for giving me the strength and perseverance needed to complete this work.

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LIST OF ABBREVIATIONS

AMP	antimicrobial peptide
BMI	body mass index
BV	bacterial vaginosis
CAMP	cathelicidin gene
CRP	C-reactive protein
GBS	group B streptococcus
FGF23	Fibroblast growth factor 23
HAMP	hepcidin gene
IL-6	Interleukin-6
IL-10	Interleukin-10
PTH	Parathyroid hormone
STI	sexually-transmitted infection
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor- α
UTI	urinary tract infection
VDR	vitamin D receptor
VDRE	vitamin D response elements
24-hydroxylase gene	CYP24A1
25(OH)D	25-hydroxyvitamin D or calcidiol
1- α -hydroxylase gene	CYP27B1
1,25(OH) $_2$ D	1,25-dihydroxyvitamin D or calcitriol

CHAPTER 1

INTRODUCTION

Specific Aims

Vitamin D insufficiency (25(OH)D <20 ng/mL) is a major public health concern for those who are unable to synthesize enough vitamin D from sun exposure and those who do not meet the dietary recommendations. Health disparities exist among pregnant adolescents and minorities with both groups being particularly vulnerable to D insufficiency, sexually-transmitted infections (STIs), and preterm birth (<37 weeks). In addition to its role in bone development, vitamin D has been linked to numerous immunological functions and disease-states. Currently, insufficient information is available on the bioactive nutrient, vitamin D, in relation to inflammatory processes and maternal/neonatal health outcomes. Recent data on the mechanisms through which vitamin D impacts inflammatory and immune mediators at the cellular-level (macrophages, dendritic cells, T cells, and B cells) provides biological plausibility for the associations which this study seeks to elucidate in group of pregnant adolescents (n=158, 13-18 years old) who completed a prospective study on adolescent pregnancy and fetal bone health. Therefore, there is a need to better understand the non-classical functions of vitamin D in preventing adverse health outcomes in high-risk populations like pregnant adolescents. To further our understanding of the relationship between vitamin D, infection, and inflammation during pregnancy, this research project addressed three goals: 1) To determine the prevalence of diagnosed infections across pregnancy and identify potential risk factors of infections; 2) To explore the relationship between systemic inflammatory biomarkers, vitamin D status, and infections; and 3) To elucidate factors associated with placental antimicrobial peptide expression and determine the impact of vitamin D

on its expression.

The specific aims and hypotheses are:

I: To assess the prevalence of maternal and placental infections and assess modifiable and non-modifiable risk factors that may be associated with these conditions.

Hypothesis: Inadequate dietary intake, lower socio-economic status, and poor health behaviors will be associated with increased odds of having clinically diagnosed infections across pregnancy.

II: To evaluate relationships between biomarkers of inflammation and vitamin D status measured across pregnancy and to identify associations between vitamin D status and clinically diagnosed infections in a group of racially and ethnically diverse pregnant adolescents.

Hypotheses: 1) Maternal vitamin D inadequacy ($25(\text{OH})\text{D} < 20 \text{ ng/ml}$) will be associated with increased infection prevalence and an increased concentration of pro-inflammatory biomarkers in the mother. 2) Cord pro-inflammatory cytokine concentrations will be positively correlated with maternal inflammatory biomarkers and placental infections and inversely associated with cord vitamin D sufficiency ($25(\text{OH})\text{D} \geq 20 \text{ ng/ml}$).

III: To elucidate factors associated with placental antimicrobial peptide (AMP) expression and to evaluate the degree to which vitamin D has an impact on placental AMP expression.

Hypothesis: Placental cathelicidin (CAMP) (transcript and protein) and hepcidin (HAMP) (transcript) will be positively associated with maternal vitamin D sufficiency ($25(\text{OH})\text{D} \geq 20 \text{ ng/ml}$).

Adolescent Sexual and Reproductive Health

Improving the sexual and reproductive health of adolescents is a major public health priority. Sexual and reproductive health outcomes for this population are known to be greatly influenced by several factors, including age of onset for sexual activity, biological maturity, contraception use, and the burden of sexually transmitted infections.(1) In the U.S., 47% of high school students (9th-12th grade) have reported engaging in sexual activity at least once, with a higher prevalence among African Americans and Hispanics.(2) Younger adolescents who engage in sexual intercourse are less likely to use contraceptives (3) which puts them at greater risk of becoming pregnant or contracting a sexually transmitted infection (STI). Other risk-taking behaviors, such as illicit drug and alcohol use, are known to steadily increase across the ages of 12-17 years.(4) This trend may pose a significant public health problem as a study in U.S. adolescents has found associations between substance abuse and increased self-report of risky sexual behaviors.(5)

Despite the decline in teen births in the US over the past 20 years (6), the teen pregnancy rate remains the highest among developed countries (57 pregnancies per 1,000 females ages 15-19 y) (7). Racial and ethnic disparities exist with Hispanic and African-Americans comprising the majority of the pregnant adolescent population (8). Pregnant teens are more at risk for adverse birth outcomes, such as low birth weight (< 2500 g), preterm birth (< 37 weeks), and neonatal mortality (9).

Pregnant adolescents are faced with a unique nutritional challenge (10). Pregnancy is a state of increased nutrient demands, and because nutrient demands are higher to support the growing adolescent, there may be competition for nutrients between the mother and fetus (10). Using the dietary reference intakes (DRIs), research has shown that nutrient intakes for pregnant

adolescents typically fall below the recommended levels for iron, calcium, vitamin E, magnesium, and vitamin D (10, 11). Adequate nutrition is not only necessary to support normal growth and development, but also to maintain an optimal immune response (12).

Infections during Pregnancy

During pregnancy, the amniotic cavity has traditionally been thought to be sterile (13). However, new data demonstrate that bacteria are capable of entering the amniotic fluid via several mechanisms: 1) ascension from the vagina to the cervix, 2) accidental needle contamination during amniocentesis or chorionic-villus sampling 3) blood traveling through the placenta (referred to as hematogenous infection), or 4) migrating through the fallopian tubes to the abdominal cavity (14). In addition to the amniotic fluid, there are several other potential sites for bacterial infections within the uterus, such as between maternal and fetal membranes (choriodecidual infection), within fetal membranes (chorioamnionitis), or the umbilical cord (funisitis) (14). Intra-amniotic infections can lead to severe consequences for fetus, such as a bloodstream infection known as sepsis (15). Intrauterine infections account for about 40% of preterm births (< 37 weeks) (16). Preterm birth occurs in about 11.4% of the U.S. pregnant female population (17) and is one of the leading causes of infant mortality in the U.S. (18). The underlying mechanisms responsible for the association between maternal infections and preterm birth are not well understood, but the activation of inflammation mediators (i.e. cytokines) (**Figure 1.1**) in response to microbial invasion has been hypothesized as one possible explanation (13, 14).

Figure 1.1: Pathogenesis of Chorioamnionitis: Maternal and Fetal Response and Complications¹

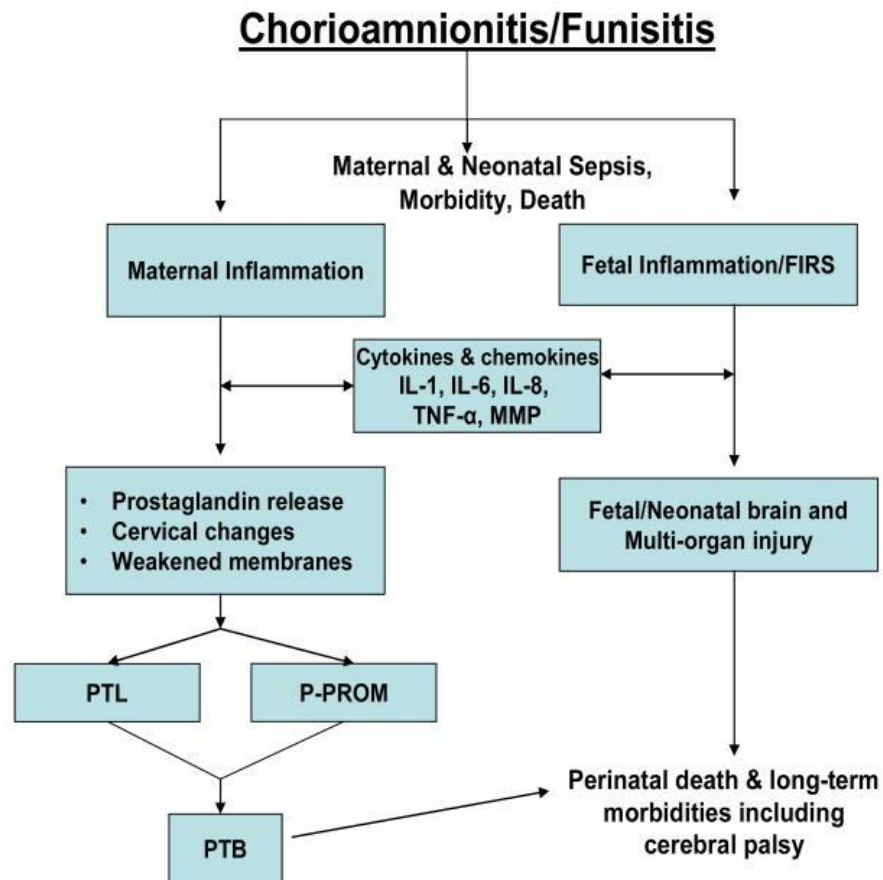


Figure source: Tita ATN, Andrews WW. Diagnosis and Management of Clinical Chorioamnionitis. *Clinics in Perinatology* 2010;37(2):339-354.⁽¹⁵⁾

¹IL=Interleukin, TNF=Tumor Necrosis Factor, MMP=Matrix metalloproteinase, FIRS=Fetal Inflammatory Response Syndrome, PTB Preterm birth

Women are usually screened for urinary tract infections (UTIs) early in pregnancy (19). UTIs can either be classified as asymptomatic bacteriuria (ASB) or symptomatic (acute cystitis or acute pyelonephritis) (20). A variety of bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and group B streptococci) can cause UTIs (20). Due to the physiological

changes associated with pregnancy, the ascension of bacteria during UTIs and subsequent development of a kidney infection (pyelonephritis) is a major concern (20).

Bacterial and yeast infections commonly occur during pregnancy. Bacterial vaginosis (BV) and candida are two of the most common types of vaginitis (21). The normal vaginal flora consists of bacteria and yeast but infection occurs when there is an overgrowth of either species. BV infection is of concern during pregnancy because of observational studies which link BV to preterm birth (22, 23).

Maternal sexually-transmitted infections (STIs) are known to cause a number of complications in the neonate, including neurological disorders, eye and lung infections, and low birth weight (LBW < 2500 g) (24). Because of the potential for maternal and neonatal complications to be associated with infections during pregnancy, the Center for Disease, Control, and Prevention (CDC) (**Table 1.1**) has established screening guidelines for several infections, especially STIs during pregnancy.

Table 1.1: CDC Screening Guidelines for Infections during Pregnancy^(24, 25)

Infection	CDC Recommendation
Chlamydia	<p><i>First prenatal visit:</i> Screen all pregnant women <25 years of age and also older pregnant women at increased risk for infection.</p> <p><i>Third trimester:</i> Rescreen if <25 years of age or at continued high risk.</p>
Gonorrhea	<p><i>First prenatal visit:</i> Screen all pregnant women <25 years of age in addition to older pregnant women who are at increased risk for infection.</p> <p><i>Third trimester:</i> Rescreen women at continued high risk.</p>

Table 1.1 cont'd

Syphilis	<i>First prenatal visit:</i> Screen all pregnant women. <i>Early third trimester:</i> Re-screen women who are high risk.
Human Immunodeficiency virus (HIV)	<i>First prenatal visit:</i> Screen all pregnant women. <i>Third trimester:</i> Rescreen women at high risk for acquiring HIV infection.
Hepatitis B (HBV)	<i>First prenatal visit:</i> Screen all pregnant women. <i>Third trimester:</i> Test those who were not screened prenatally, those who engage in behaviors that put them at <i>high risk</i> for infection, and those with signs or symptoms of hepatitis at the time of admission to the hospital for delivery.
Hepatitis C (HCV)	<i>First prenatal visit:</i> Screen all pregnant women at increased risk.
Human Papillomavirus (HPV)	There are no screening recommendations.
Herpes (HSV)	Evidence does not support routine HSV-2 serologic testing among asymptomatic pregnant women.
Bacterial Vaginosis (BV)	Evidence does not support routine screening in asymptomatic pregnant women at high or low risk for preterm delivery.
Trichomoniasis	Evidence does not support routine screening for in asymptomatic pregnant women.

Table adapted from CDC. STDs during Pregnancy - CDC Fact Sheet.

Pregnancy is considered a state of altered immunology with T lymphocyte (cell) and natural killer cell activity being reduced, while phagocytic activity and the number of dendritic cells, neutrophils, monocytes, and regulatory T cells being maintained or enhanced (26). These immunological changes might explain why infection susceptibility and severity increase across pregnancy (26). Vitamin D is not only capable of promoting bone health but it's also known to play a role in modulating the innate and adaptive immune response to infection. Despite these findings, limited data exist on risk factors for infection in adolescent pregnant populations and the role of vitamin D in regulating immunological activity in response to various infections occurring during pregnancy.

Sources of Vitamin D

Vitamin D is a unique, bioactive nutrient which can be produced in the skin (as vitamin D₃) from 7-dehydrocholesterol following exposure to UVB light. The photo-isomerization of pre-vitamin D₃ is followed by thermal isomerization into vitamin D₃ (27). The dermal synthesis of vitamin D is affected by the season of the year. In periods of high sun exposure in the summer, the UVB radiation is of greater intensity. However, those living at northern latitudes (above 42 degrees) receive much lower UVB exposure during the winter season (28) and do not receive sufficient UVB exposure for vitamin D synthesis. Individual factors associated with reduced vitamin D synthesis include older age, sunscreen use, and darkly pigmented skin (29). In addition to endogenous production, vitamin D can also be consumed in the diet from plant sources as vitamin D₂ (ergocalciferol), from animal sources as vitamin D₃ (cholecalciferol) or from dietary supplements (either as D₂ or D₃). Few naturally occurring sources of dietary vitamin D are available, and these include fatty fish, egg yolk, and fish liver oil (29). Fortification of

foods (such as milk and breakfast cereals) provides the major source of vitamin D found in in the US diet (30). Dietary vitamin D supplements usually contain a dose of 400 IU/day, but supplemental D content has been increasing over the years. Based on a recent review published by the Institute of Medicine (IOM), in the US, supplemental vitamin D can be ingested in multi-vitamin/multi-mineral formulations or as a single supplement with up to 5,000 IU of vitamin D₃ per dose or 50,000 IU of vitamin D₂ per dose (with a prescription) (29).

Vitamin D Metabolism and Homeostasis

Vitamin D is a fat-soluble nutrient which is absorbed with other dietary fats in the small intestine (31, 32) and it enters the small intestine either by passive diffusion or transport-mediated uptake via the scavenger receptor class B type 1 (SR-B1) or NPLC1 (33). Following absorption, vitamin D is packaged with cholesterol, triglycerides, and other lipids into chylomicrons (29) which transport this prohormone to the lymph, periphery, and finally to the liver as chylomicron remnants (34). The 25-hydroxylation step is loosely regulated by the amount of baseline 25(OH)D circulating in the body (35). Vitamin D from endogenous synthesis or dietary intake is either hydroxylated by the 25-hydroxylase enzyme (CYP2R1) which converts it into the prohormone 25-hydroxyvitamin D (25(OH)D) in the liver or vitamin D can be stored in adipose tissue (34). The sequestration of vitamin D in adipose tissue is associated with lower 25(OH)D levels (36). Once 25(OH)D leaves the liver and enters the circulation, it is bound by the vitamin D-binding protein (DBP) (34). In the kidney and in other tissues, the uptake (endocytosis) of 25(OH)D bound to DBP is facilitated by the proteins megalin and cubilin (37, 38). Once unbound from DBP, 25(OH)D is converted by the 1- α -hydroxylase (also known as CYP27B1) into its biologically active form 1,25-dihydroxyvitamin D (1,25(OH)₂D) (also known

as calcitriol) (34) in the mitochondria. The prohormone 25(OH)D serves as the best biomarker of vitamin D status because it has a longer serum half-life than calcitriol (2-3 weeks vs. ~ 8 hours), it reflects both endogenously synthesized vitamin D and that obtained from dietary sources, its circulating concentration is 1000 times greater than calcitriol's, and its production is not tightly regulated (39).

The homeostatic mechanisms that control serum concentrations of calcitriol, calcium, and phosphate are important in bone development (**Figure 1.2**). Calcium and phosphate are very important components of bone, and calcium can be absorbed both passively and actively. Serum concentrations of calcium are tightly regulated by three hormones: calcitriol, parathyroid hormone (PTH), and calcitonin. Calcitriol can increase serum calcium concentrations in three ways. First, calcitriol acts directly on the small intestine to stimulate calcium absorption as well as phosphate absorption independently (29). With the help of the calcium sensing receptor in the parathyroid glands, low serum calcium leads to the production of PTH. Calcitriol production is increased by PTH to stimulate the secretion of the receptor activator of nuclear factor kappa-B ligand (RANK-L) which is necessary for osteoclast formation and subsequent bone resorption (29). Finally, PTH can act directly on the kidney to increase calcium reabsorption (40). Under states of high serum calcium, calcitonin functions to oppose the effects of PTH and promote the utilization of calcium by bone (29).

Figure 1.2: The Classical Effects of Vitamin D and Its Metabolic Pathways

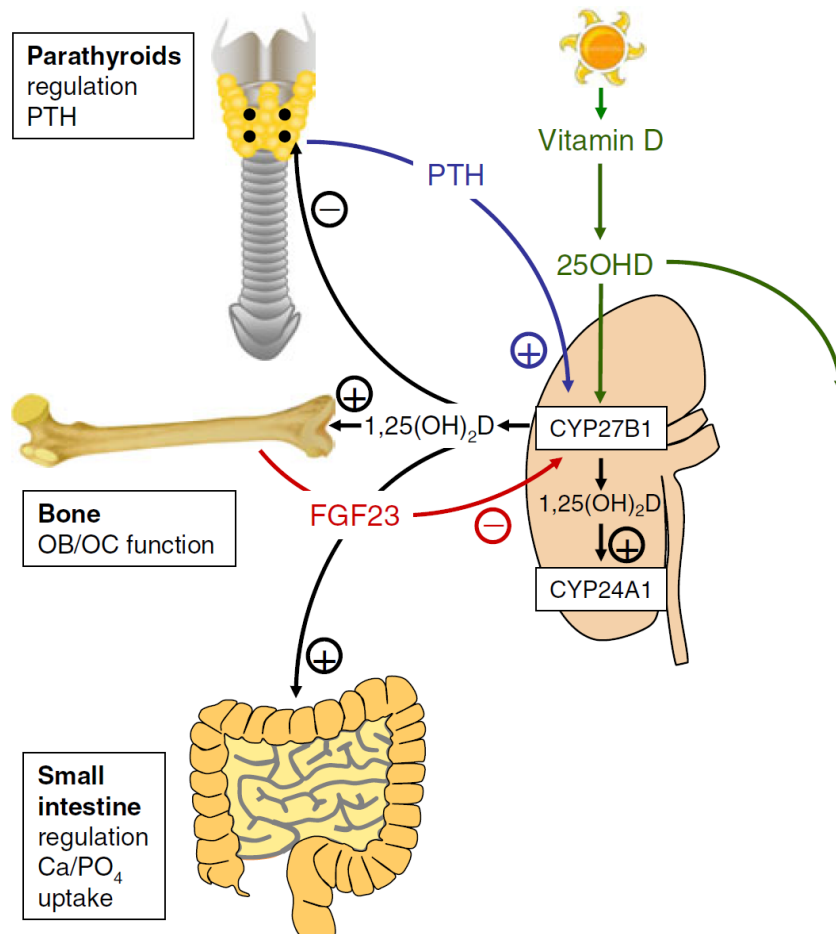


Figure source: Hewison, M., Vitamin D and immune function: an overview. *Proc Nutr Soc*, 2012. **71**(1): p. 50-61.⁽⁴¹⁾

Phosphorus homeostasis was previously thought to only be regulated by the activity of PTH and calcitriol as side consequences of the regulation of calcium homeostasis, but beginning in 2000, fibroblast growth factor 23 (FGF23) was discovered as an additional hormone involved in phosphorus homeostasis (42, 43). PTH and FGF23 are not only involved in the maintenance of calcium and phosphorus homeostasis (29) but also in regulating calcitriol production. PTH and FGF23 (44) are known to induce and inhibit CYP27B1 activity, respectively (**Figure 1.3**). The 24-hydroxylase enzyme (also known as CYP24A1) regulates the catabolism of both calcitriol

and 25(OH)D into inactive metabolites in the kidney (35). The CYP24A1 enzyme can be induced by FGF23 and inhibited by PTH (45). Recently, there has been an increase in research addressing CYP24A1 expression as it has been suggested that elevated or abnormally long-lasting expression of this enzyme may underlie diseases which would normally be responsive to treatment with vitamin D, such as FGF23-dependent renal phosphate wasting disorders (46).

Figure 1.3: FGF23 and PTH Maintain Phosphate Homeostasis

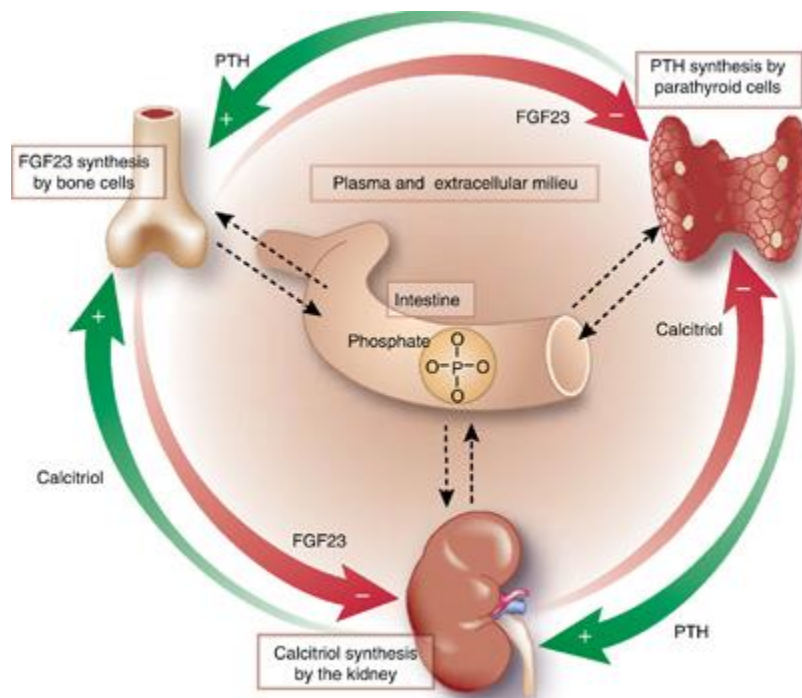


Figure source: Torres, P.A. and D.P. De Brauwere, Three feedback loops precisely regulating serum phosphate concentration. *Kidney Int*, 2011. **80**(5): p. 443-5.⁽⁴⁷⁾

Calcitriol acts by binding to the vitamin D receptor (VDR), which heterodimerizes with retinoid-X receptor (RXR) to activate approximately 3% of the human genome for genes that contain vitamin D response elements (VDREs) (48). Under conditions of elevated calcitriol, CYP24A1 is up-regulated through the VDRE (35) and CYP27B1 is down-regulated through a

VDRE (49). Vitamin D metabolism can also be affected by genetic polymorphisms in VDR (50, 51), CYP27B1, CYP24A1 (52, 53), DBP (54), CYP2R1 (55) and 7-dehydrocholesterol reductase (DHCR7) (56).

Because various immune cells (T cells, B cells, NK cells, and monocytes) contain the VDR, vitamin D is thought to regulate their function (57). Inadequate vitamin D status has been associated with a variety of acute and chronic diseases, such as acute respiratory tract infections (58), urinary tract infections (59), rheumatoid arthritis (60), type I diabetes (61), cancer (62), multiple sclerosis (63), and systemic lupus erythematosus (64). However, limited data from randomized controlled trials are available and observational data on the relationship between vitamin D and extra-skeletal diseases are inconsistent and inconclusive (65).

It is well known that the active form of vitamin D, calcitriol, plays an important role in maintaining calcium and phosphate homeostasis and regulating bone metabolism. Severe vitamin deficiency may result in conditions characterized by inadequate bone mineralization in children (rickets) and adults (osteomalacia) (34). Therefore, the dietary requirements for vitamin D established by the Institute of Medicine (IOM) in 2011 were developed based solely on the nutrient's role in promoting bone health. These recommendations reflect the amount of dietary vitamin D needed to achieve adequate 25(OH)D serum concentrations assuming minimal sun exposure (**Table 1.2**). An Estimated Average Requirement (EAR) and Recommended Dietary Allowance (RDA) have been established for all age groups except for infants 0-12 months old (29). Due to limited data on the infant population, an Adequate Intake (AI) of 400 IU for this age group has been defined in place of an EAR (29).

Table 1.2: Institute of Medicine Serum 25(OH)D cutoffs ⁽²⁹⁾

“At risk of deficiency”	“At risk for inadequacy”	“Sufficiency”	“There may be a reason for concern”
25(OH)D < 12 ng/ml (30 nmol/L)	25(OH)D 12 to 20 ng/ml (30-50 nmol/L)	25(OH)D ≥ 20 ng/ml (50 nmol/L)	25(OH)D >50 ng/ml (125 nmol/L)

Vitamin D during Pregnancy

During pregnancy, maternal ionized serum calcium concentrations remain constant but total calcium concentrations drop slightly during starting in early pregnancy (66). The regulation of PTH during pregnancy remains controversial. Some research has suggested that PTH decreases in pregnancy except among populations in Asia or Gambia with lower calcium intake (66). However, recent studies conducted in U.S. pregnant women (N= 350) and adolescents (N=168) ingesting high calcium intakes between 900 - 1,000 mg/d have shown that PTH increases during pregnancy in up to 25% of women, particularly in those with limited calcium intakes and low vitamin D status (67, 68). Because intestinal calcium absorption doubles in the mother during pregnancy, it is thought to be the main mechanism used to meet fetal calcium demands (66). Due to increases in calcium absorption and calcitonin during pregnancy, renal calcium excretion increases during pregnancy which may lead to hypercalciuria (66).

Recently, researchers have found that during pregnancy, vitamin D is also essential for fetal skeletal development (69-71). In addition, vitamin D may have other roles in fetal developmental programming *in utero* for diseases later in life such as allergies (72) and cardiovascular disease (73) which are areas of growing research interest.

During pregnancy, there are several additional factors to consider in vitamin D

metabolism and maternal and fetal vitamin D kinetics. First, the fetus is unable to synthesize vitamin D and must rely solely on the maternal supply of 25(OH)D which readily crosses the placenta (74, 75). Because calcitriol does not readily cross the placenta (76, 77), and fetal PTH concentrations are low (67), cord concentrations of calcitriol tend to be lower than those measured in maternal serum (74, 77). In addition to low PTH concentrations, high serum phosphorus and calcium concentrations also contribute to lower fetal calcitriol concentrations as these factors suppress renal CYP27B1 expression in the fetus (29). The mother and the fetus are able to independently synthesize the active metabolite of vitamin D from 25(OH)D. Recent research has indicated that the placental expression of megalin and cubilin increases across gestation and that these receptors may be involved in the placental transfer of 25(OH)D at this maternal-fetal interface (78). Second, serum calcitriol concentrations are elevated during pregnancy (79). Total calcitriol concentrations are known to double or triple during early pregnancy while 25(OH)D concentrations remain constant (80). The rationale for why serum calcitriol concentrations are not tightly regulated during pregnancy compared to during non-pregnant state is unknown.

Over 30 years ago the placenta was identified as a major extra-renal site for vitamin D metabolism as the placenta can convert the pro-hormone 25(OH)D into calcitriol (81, 82). The placenta expresses all of the enzymes necessary for vitamin D metabolism and regulatory function: CYP27B1, CYP24A1 and the VDR. Vitamin D is thought to regulate hormones involved in the maintenance of pregnancy and placental physiology with studies demonstrating an effect of 1,25(OH)₂D on estradiol (83), human placental lactogen (hPL) (84), and human chorionic gonadotropin (hCG) (85). Some research suggests that the placental CYP24A1 gene is methylated and unresponsive to 1,25(OH)₂D (86). Therefore, placental CYP27B1-mediated

conversion in the absence of CYP24A1-mediated catabolism is thought to provide for unrestricted placental synthesis of 1,25(OH)₂D during pregnancy (86). However, others have found significant associations between placental CYP24A1 and CYP27B1 expression (76), which suggest that CYP24A1 may not be silenced during pregnancy. Although the placenta is not thought to be the source of elevated maternal circulating concentrations of 1,25(OH)₂D, a recent study has shown a significantly positive association between placental CYP27B1 mRNA expression and maternal 1,25(OH)₂D at mid-gestation (76). Although the biological function of increased calcitriol during pregnancy is not yet known, researchers have proposed that placental calcitriol exhibits an autocrine/paracrine function (87) and this effect is immune-modulatory (88).

Vitamin D insufficiency during pregnancy has been associated with adverse maternal and neonatal outcomes. Observational data suggests a relationship exists between low 25(OH)D concentrations and an elevated risk of preeclampsia (89), bacterial vaginosis (90), Cesarean section (C-section) (91), and babies born small-for-gestational age (SGA) (92). Recent studies have shown that oral vitamin D supplementation increases both 25(OH)D and 1,25(OH)₂D during pregnancy (68, 93) and 25(OH)D \geq 40 ng/ml is needed to support maximum systemic 1,25(OH)₂D production (68). However, improvements in maternal/fetal outcomes, such as pre-eclampsia (93), gestational age at birth (68), or neonatal birth weight (68), as a direct result of supplementation remain inconclusive. Additional sufficiently powered RCTs in pregnant women are needed to further elucidate the findings between low serum 25(OH)D and adverse pregnancy outcomes evident in observational studies.

Despite the potential impact of vitamin D on maternal and neonatal outcomes, the EAR (400 IU) and RDA (600 IU) for vitamin D do not differ between non-pregnant and pregnant

females. Data from National Health and Nutrition Examination Survey (NHANES) 2001-2006 demonstrate that approximately 20% of U.S. pregnant women are not vitamin D sufficient (25(OH)D \geq 20 ng/ml) (**Table 1.3**). A separate analysis of the NHANES data demonstrated that compared to pregnant Caucasian females (31 ng/ml), the mean 25(OH)D concentrations was almost 30% lower in pregnant Hispanic females (22 ng/ml) and approximately 50% lower in pregnant African-American females (16 ng/ml) (94).

Table 1.3: Population Survey Data from Pregnant/Lactating or Reproductive-age women, U.S.⁽⁶⁶⁾

Group	Mean 25(OH)D (ng/ml)	Range 5 th -95 th percentile (ng/ml)
Pregnant or lactating ¹	25	9.8-43.6
Age 14-18	22.9	8.3-41.6
Age 19-30	22.4	7.4-44.4
Age 31-50	22.1	7.7-40.4

Table adapted from Kovacs CS. The role of vitamin D in pregnancy and lactation: insights from animal models and clinical studies. *Annu Rev Nutr* 2012;32:97-123.

Original data was obtained from the National Health and Nutrition Examination Survey. (NHANES) 2001-2006 using the Diasorin radioimmunoassay (RIA).

¹Among pregnant or lactating women (n=1,067), 72% had 25(OH)D \geq 20 ng/ml, 21% 12-19 nmol/L, and 7% < 12 ng/ml

Adolescents are at high-risk for both vitamin D insufficiency (94) and sexually-transmitted infections (95) and once they become pregnant, they are also at high risk for adverse birth outcomes (9). Therefore, the current vitamin D recommendations may not be adequate for minimizing adverse immunological or other non-bone health mediated effects during adolescent pregnancy which may be detrimental to the mother and/or the fetus (**Tables 1.4-1.7**), especially among high-risk populations such as pregnant minority adolescents.

Table 1.4: Summary of Research on Vitamin D and Pre-eclampsia^(89, 96, 97)

Study	Subject Characteristics	Results
Bodnar et al. (2007)⁽⁸⁹⁾ (Observational- case-control) N=274 Serum 25(OH)D measured with a commercial ELISA from Immunodiagnostic Systems Limited (IDS, UK) and validated against a HPLC method.	Pregnant women in America (14 - 44 y) at \leq 16 weeks gestation	<ul style="list-style-type: none"> • Mean maternal serum 25(OH)D was significantly lower in women who developed pre-eclampsia ($P < 0.01$) • A 50 nmol/L decrease in serum 25(OH)D concentration doubled the likelihood of preeclampsia (OR: 2.4, 95% CI, 1.1–5.4)
Bodnar et al. (2014)⁽⁹⁶⁾ (Observational- case-cohort) N=3,703 Serum 25(OH)D measured via liquid chromatography-tandem mass spectrometry.	Pregnant women in America (age range: N/A) with serum 25(OH)D measured at \leq 26 weeks gestation.	<ul style="list-style-type: none"> • Maternal serum 25(OH)D \geq 50 nmol/L was associated with a 35% reduction in the risk of pre-eclampsia (adjusted RR: 0.65, 95% CI, 0.43–0.98) compared to those with 25(OH)D $<$ 50.
Naghshineh et al. (2016)⁽⁹⁷⁾ (RCT) N=140 Treatment: 600 IU/d of vitamin D until parturition Control: placebo	Pregnant women (mean age: 25 y) in Iran at $<$ 16 weeks gestation.	<ul style="list-style-type: none"> • No significant difference in number of women diagnosed or in the severity of pre-eclampsia between the 2 study groups ($P = 0.09$).

Enzyme-linked immunoassay; ELISA, High Performance Liquid Chromatography; HPLC, Odds Ratio; OR, Confidence Interval, CI, Relative Risk; RR, Randomized Controlled Trial; RCT.

Table 1.5: Summary of Research on Vitamin D and Preterm Birth (< 37 weeks)^(98, 99)

Study	Subject Characteristics	Results
Bodnar et al. (2015)⁽⁹⁸⁾ (Observational- case-cohort) N=3,453 Maternal serum 25(OH)D was measured using liquid chromatography–tandem mass spectrometry.	Pregnant women in America (≥ 18 y) with prenatal serum samples obtained at ≤ 20 weeks gestation.	<ul style="list-style-type: none"> Preterm birth (PTB) incidence was significantly higher among those with serum 25(OH)D < 50 nmol/L (11.3%) compared to those with 25(OH)D 50-74.9 nmol/L (8.6%) or 25(OH)D ≥ 75 nmol/L (7.3%) (P<0.01).
Wagner et al. (2016)⁽⁹⁹⁾ (Post-hoc analysis of two combined vitamin D RCTs) N=509 Maternal serum 25(OH)D was measured using a rapid, direct radioimmunoassay (RIA) (Diasorin, USA). Treatment: study 1: 400 IU/d, 2000 IU/d, or 4,000 IU/d of vitamin D ⁽⁶⁸⁾ or study 2: 2,000 IU/d vs 4,000 IU/d of vitamin D ⁽¹⁰⁰⁾ .	Pregnant women in America (17 – 44 y) between 12 to 16 weeks gestation.	<ul style="list-style-type: none"> Maternal serum 25(OH)D ≥ 40 ng/ml was associated with a 57% lower risk of preterm birth when compared to those with 25(OH)D ≤ 20 ng/ml (RR: 0.43, 95% CI, 0.22-0.83). A fitted curve showed that gestational age at birth rose steadily with increasing 25(OH)D concentrations and then plateaued at 40 ng/ml.

Preterm birth; PTB, Randomized Controlled Trial; RCT, Relative Risk; RR, Confidence Interval, CI.

Table 1.6: Summary of Research On Vitamin D and Gestational Diabetes⁽¹⁰¹⁻¹⁰⁴⁾

Study	Subject Characteristics	Results
Burris et al. (2012) ⁽¹⁰¹⁾ (Observational- prospective cohort study) N=1,314 Maternal serum 25(OH)D was measured with both an automated chemiluminescence immunoassay (CLIA) a manual radioimmunoassay (RIA).	Pregnant women in America (mean age: 32 y) at < 22 weeks gestation.	<ul style="list-style-type: none"> Maternal serum 25(OH)D concentration <25 nmol/L was associated with higher odds of gestational diabetes when compared to those with 25(OH)D ≥ 25 nmol/L (OR: 2.2, 95% CI, 0.8- 5.5).
Clifton-Bligh et al. (2008) ⁽¹⁰²⁾ (Observational- prospective cohort study) N=264 Maternal serum 25(OH)D was measured with the Nichols Advantage assay (Nichols Institute Diagnostics, USA)	Pregnant women in Australia (mean age: 33 y) with blood sampling conducted at a mean of 29 weeks gestation.	<ul style="list-style-type: none"> Maternal serum 25(OH)D concentration was significantly lower in those with gestational diabetes (48.6 ± 24.9 nmol/L) vs. those without (55.3 ± 23.3 nmol/L) (P=0.04).
Farrant et al. (2009) ⁽¹⁰³⁾ (Observational- prospective cohort study) N=559 Maternal 25(OH)D was measured using a radioimmunoassay (RIA) Immunodiag. Ltd, UK	Pregnant women in India (mean age: 24 y) at < 32 weeks gestation.	<ul style="list-style-type: none"> No statistically significant associations between maternal serum 25(OH)D concentrations and incidence of gestational diabetes were evident (P=0.8).

Table 1.6 cont'd

Makgoba et al. (2011)⁽¹⁰⁴⁾
(Observational- case-control
study)

N=248

Maternal 25(OH)D was
measured by liquid
chromatography-tandem
mass spectrometry.

Pregnant women in the
United Kingdom (mean
age: 33 y) with first
trimester blood samples

- Baseline maternal serum 25(OH)D concentrations were not significantly different between those who later developed gestational diabetes and those who did not (P=0.9).

Odds Ratio; OR, Confidence Interval; CI.

Table 1.7: Summary of Research on Vitamin D and Cesarean Section^(91, 105, 106)

Study	Subject Characteristics	Results
Merewood et al. (2009)⁽⁹¹⁾ N=253 (Observational- cross-sectional study) Maternal 25(OH)D was measured with a competitive protein binding assay.	Pregnant women in America (mean age: 26 y) within 72 hours of giving birth.	<ul style="list-style-type: none"> Women with maternal serum 25(OH)D < 37.5 nmol/L were almost 4 times as likely to have a cesarean section compared to with 25(OH)D ≥ 37.5 nmol/L (OR: 3.84, 95% CI, 1.71-8.62).
Gernand et al. (2015)⁽¹⁰⁵⁾ N=2,798 (Observational- retrospective cohort study) Maternal 25(OH)D was measured by liquid chromatography-tandem mass spectrometry.	Pregnant women in America (mean age: N/A) with serum samples obtained at ≤ 26 weeks gestation.	<ul style="list-style-type: none"> No statistically significant associations between maternal serum 25(OH)D concentrations and cesarean section were evident.
Scholl et al. (2012)⁽¹⁰⁶⁾ N=1,153 (Observational- prospective cohort study) Maternal 25(OH)D was measured by HPLC (Chromosorb, Germany).	Pregnant minority women in America (mean age: 23 y) with serum samples collected at entry into prenatal care (mean: ~14 weeks gestation).	<ul style="list-style-type: none"> Maternal serum 25(OH)D < 37.5 nmol/L was associated with almost 2 times greater odds of cesarean delivery compared to those with 25(OH)D ≥ 80 nmol/L (AOR: 1.74, 95% CI, 1.13-2.67).

Odds Ratio; OR, Confidence Interval; CI, High Performance Liquid Chromatography; HPLC, Adjusted Odds Ratio; AOR.

Vitamin D and Innate Immunity

Innate immunity is a non-specific response and the first line of defense against invading microorganisms (107). This process involves biochemical and cellular defense mechanisms

which are already in place long before an infection occurs; thus, the response is rapid (107) . The major components of innate immunity are epithelial barriers and antimicrobial substances, cytokines, blood proteins involved in the complement system, phagocytic cells (neutrophils and macrophages) and natural killer (NK) cells (107). The activation of transmembrane toll-like receptors (TLRs) in monocytes, macrophages, dendritic cells as well as in several epithelial cells (i.e. intestine, bladder, and vagina) is a critical step in microbial pattern recognition and subsequent innate immune response (107). Once activated, TLRs induce antimicrobial peptides and reactive oxygen species to destroy the microorganism (108).

Antimicrobial peptides (AMPs) are usually small, amphipathic proteins found on almost all body surfaces and in every tissue (107) . AMPs such as human cathelicidin and β -defensins are known to kill a broad range of pathogens, including gram-positive and gram-negative bacteria, fungi, and viruses (109). The antimicrobial action of vitamin D is thought to be mediated through an intracrine cellular response to microbial stimulation (41). Liu and colleagues have demonstrated that bacteria and their surface-associated molecules can induce the expression of CYP27B1 and VDR mRNA in human macrophages *in vitro* (110) and in human monocytes *ex vivo* (111). The mechanism through which calcitriol regulates innate immunity is thought to be mediated by stimulation of cathelicidin production in TLR-activated monocytes and macrophages (111) (**Figure 1.4**). Because vitamin D response elements have been identified in the promoter regions of the genes encoding antimicrobial peptides such as cathelicidin and human β -defensin-2 (112), it has been suggested that vitamin D plays a role in regulating their expression. Furthermore, in response to *Mycobacterium bovis*, cathelicidin has been shown to co-localize with bacteria-containing vacuoles in monocytes treated with calcitriol but not in those without treatment (110).

Figure 1.4: Regulation of Innate Immunity by $1,25(\text{OH})_2\text{D}$ ¹

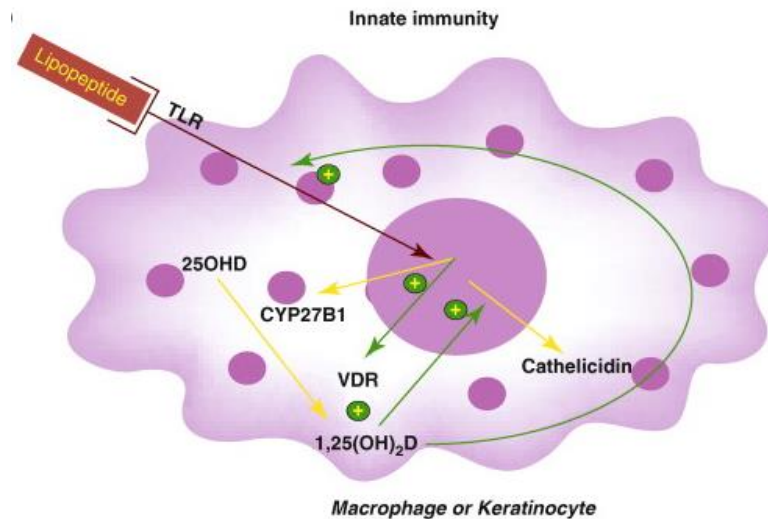


Figure Source: Bikle DD. Vitamin D: newly discovered actions require reconsideration of physiologic requirements. *Trends in Endocrinology and Metabolism* 2010; 21(6):375-384.⁽¹⁰⁸⁾

¹Yellow arrows demonstrate TLR stimulation of CYP27B1 and cathelicidin (independently) and the metabolism of 25(OH)D into $1,25(\text{OH})_2\text{D}$. Green arrows indicate upregulation of VDR by $1,25(\text{OH})_2\text{D}$ and subsequent induction of cathelicidin.

Numerous *in vitro* studies have shown that calcitriol increases the production of the antimicrobial peptides cathelicidin (110, 113, 114) and β -defensin-2 by binding to the VDR in macrophages (113, 114), one of the first *cells* to recognize and engulf foreign substances during infections. *In vitro* studies have also shown that low concentrations of 25(OH)D were associated with reduced production of cathelicidin by human macrophages in response to *Mycobacterium tuberculosis* (110). Serum 25(OH)D concentrations have been shown to be consistently lower in African-Americans when compared to Caucasians (110, 115), *ex vivo* supplementation of African-American sera with 25(OH)D to physiological concentrations (1-100 nM) has been

shown to restore cathelicidin production (110). Similarly, *in vivo* supplementation (50,000 IU of vitamin D₂) of elderly patients with 25(OH)D <30 ng/ml has been shown to significantly enhance monocyte production of cathelicidin in monocytes isolated from peripheral blood mononuclear cells (PBMCs) (111). AMPs are known to be expressed in human trophoblasts (116, 117). However, the role of placental antimicrobial peptides and vitamin D in the prevention of localized infections during pregnancy remains unclear.

Hepcidin is a well-recognized AMP which functions to limit extracellular microbial growth by preventing the release of intra-cellular iron, the most essential growth-limiting nutrient for potential pathogens (120). The gene for hepcidin (HAMP) contains a VDRE (118). Recent research has shown that hepcidin is also responsive to calcitriol bound to the VDR (118). In this study, 25(OH)D and calcitriol administered to PBMC monocytes decreased hepcidin mRNA expression *in vitro*, and vitamin D supplementation (100,000 IU vitamin D₂) led to a ~30% decrease in circulating hepcidin levels in healthy adults (*in vivo*) within 24 hours (118). The opposing action of calcitriol on hepcidin may reflect a delicate balance during intracellular infections as iron plays a key role in promoting both host antimicrobial activity (as an essential co-factor for macrophages) and intracellular pathogen survival (119). Free or non-transferrin bound serum iron is important for extracellular microbial growth (120). However, it is not well understood how calcitriol might differentially regulate hepcidin in response to intracellular vs extracellular microbial stimuli.

Research studies have indicated that microorganisms may escape the host defense by reducing AMP activity (121) and the effect of vitamin D in innate and adaptive immunity. *M. tuberculosis*, *M. leprae*, and human immunodeficiency virus (HIV) have been shown to inhibit VDR activity (122).

Cytokines play a role in both innate and adaptive immunity (107). During innate immunity, activated macrophages secrete cytokines which increase the permeability of blood vessels and chemokines which attract other immune cells, such as neutrophils (107). The accumulation of immune cells at the site of an infection can lead to tissue injury in a process known as inflammation (107). Numerous *in vitro* studies have shown that vitamin D inhibits pro-inflammatory cytokine production (43, 123, 124) while increasing anti-inflammatory cytokines in human immune cells (125, 126).

Vitamin D and Adaptive Immunity

Adaptive (or “acquired”) immunity is a specific, adaptive response to an infection (107) . This process involves remembering a prior exposure and developing specificity for the unique microbial molecules; thus, the response to repeated exposures is more rapid (107) . Lymphocytes (T cells and B cells) and the products they secrete (e.g. antibodies) are the major components of adaptive immunity (107) . The initiation of adaptive immunity requires antigen-presenting cells (e.g. dendritic cells) to capture and “present” antigens to specific lymphocytes (107) .

Vitamin D plays a role in regulating the antigen presentation process which is necessary to activate adaptive immunity. Both the VDR (127) and CYP27B1 enzyme (128) are expressed in dendritic cells (DCs). Studies have shown that 25(OH)D (*in vitro*) (128) and calcitriol (*in vitro and in vivo*) act to suppress DC maturation and function (128-130) with VDR and CYP27B1 knockout mice displaying an increase in mature DCs (131, 132). Calcitriol is thought to function via a paracrine mechanism in DCs (41). Immunosuppression occurs as VDR-poor DCs produce calcitriol which acts on VDR-rich DCs to suppress their maturation and subsequent ability to activate T cells (41).

Both T cells and B cells express the VDR (133, 134). In cell-mediated immunity, naive CD4⁺ T helper cells may differentiate into two types of cells: Th1 cells or Th2 cells (107). Th1 cells are involved in promoting the inflammation response by producing pro-inflammatory cytokines which stimulate macrophage activation (e.g. INF- γ , TNF- α , IL-2) (107). During inflammation, IL-6 is capable of inducing both C-reactive protein (CRP) and hepcidin production in the liver. In contrast, Th2 cells are responsible for inhibiting macrophage activation by producing anti-inflammatory cytokines (e.g. IL-4, IL-5, and IL-13) (107). In T helper cells, calcitriol acts to inhibit Th1 cells *in vitro* (135) and enhance the production of Th2 cell-associated cytokines *in vitro* (136, 137). It is not well-understood if the shift from Th1 to Th2 cells is present *in vivo* (41) as results on this topic are conflicting (138). Despite this, calcitriol is believed to function in maintaining a balance between inflammation and immunosuppression (41). Th17 cells are another class of lymphocytes which are responsible for promoting a pro-inflammatory cytokine (IL-17) response to microbial invasion (41), and they have been associated with inflammatory tissue damage (139, 140). *In vitro* studies have shown that calcitriol suppresses Th17 development (141, 142) and inhibits IL-17 production (28). Similarly, *in vivo* studies have demonstrated elevated IL-17 levels in CYP27B1 knockout mice (138). Additionally, calcitriol has been shown to induce the development of suppressor or regulatory T-cells (Tregs) leading to enhanced IL-10 production (143-145). The function of calcitriol in CD8⁺ T cells remains unclear (41). In B cells, calcitriol is believed to act in an autocrine/intracrine mechanism (41) as it has been shown that B cells contain CYP27B1 (146), and calcitriol is capable of inhibiting B cell differentiation, proliferation, and immunoglobulin production (146).

Because many pathogenic intracellular microorganisms have evolved to evade host

defense mechanisms, they can persist and cause chronic T cell and macrophage activation (107). The accumulation of immune cells (especially macrophages) at the site of an infection can lead to tissue injury: the formation of granulomas which are associated with necrosis (un-programmed cell and tissue death) and fibrosis (excess deposition of fibrous connective tissue/scarring) (107). To prevent the potentially adverse effects of persistent immune signaling, the function of vitamin D in adaptive immunity is thought to be immunosuppression (41) (**Figure 1.5**).

Figure 1.5: Regulation of Adaptive Immunity by $1,25(\text{OH})_2\text{D}^1$

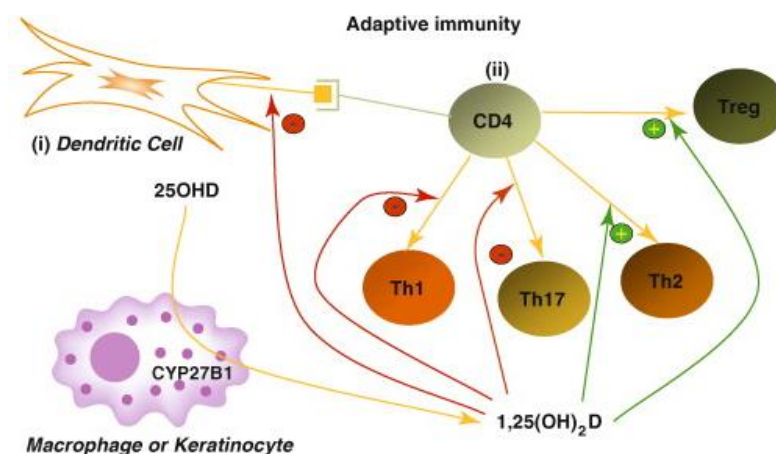


Figure Source: Bikle DD. Vitamin D: newly discovered actions require reconsideration of physiologic requirements. *Trends in Endocrinology and Metabolism* 2010;21(6):375-384.⁽¹⁰⁸⁾

¹Yellow arrows demonstrate dendritic cell stimulation of CD4 cell differentiation into four types (Th1, Th17, Th2, and Treg) and the metabolism of 25(OH)D into $1,25(\text{OH})_2\text{D}$. Green arrows indicate promotion and red arrows indicate inhibition of cell differentiation by $1,25(\text{OH})_2\text{D}$, respectively.

Data on the regulation of inflammation during pregnancy are both limited and conflicting. Calcitriol has been shown to downregulate the mRNA expression of the pro-inflammatory cytokines TNF- α and IL-6 in cultured placental cells from preeclamptic women

(87), and a recent RCT conducted in 57 US pregnant women found that 2000 IU of vitamin D supplementation was more effective in increasing IL-10⁺ regulatory CD4⁺ T cells than supplementation with 400 IU (147). In contrast to vitamin D's well-known anti-inflammatory action, calcitriol has been shown to down-regulate the mRNA expression of anti-inflammatory cytokine IL-10 in cultured human trophoblasts under experimental conditions with or without inflammation (148).

During pregnancy, Th2 cell induction is critical to promote a normal pregnancy (115). If the implantation of the fetus is not tolerated, an adverse Th1 pro-inflammatory cytokine response is thought to be initiated (149). Vitamin D is believed to play a role in the shift to a Th2 anti-inflammatory immune response during pregnancy (115). Because microbial infection during pregnancy leads to the production of pro-inflammatory cytokines, research has suggested that the cascade of pro-inflammatory cytokines and prostaglandin production may play a role in the development of preterm birth (<37 weeks of gestational age) associated with maternal infections (150). Due to the established anti-inflammatory activity of calcitriol, it is important to understand the vital role vitamin D may play in regulating the immune response during infection and inflammatory states which arise throughout pregnancy.

Parent Study

Pregnant adolescents (n=158, aged ≤18 y) were recruited from the Rochester Adolescent Maternity Program (RAMP) clinic in Rochester, NY and longitudinally followed across pregnancy. An additional 10 adolescents were recruited from Baltimore but did not have inflammatory indicators measured thus the group studied in Rochester will be used for this research. The parent study was a study of maternal and fetal bone health and iron status across

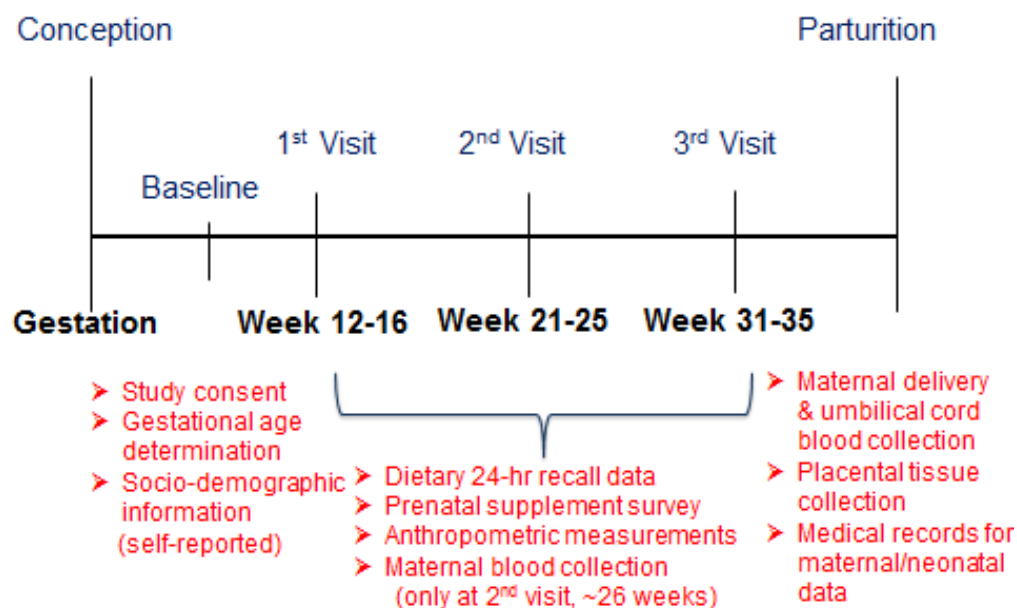
gestation. The study recruitment period was undertaken between 2006 - 2009. Non-smoking adolescents between 12 and 30 weeks of gestation at entry were eligible to participate in the study if they were carrying a single fetus and did not have a preexisting medical complication (e.g. HIV infection, diabetes/gestational diabetes, elevated diastolic blood pressure (>110), pregnancy-induced hypertension, malabsorption diseases, or diagnosed eating disorders). Teens with a history of steroid use and/or substance abuse, those taking medications known to influence calcium or vitamin D homeostasis, and those identified as having elevated blood lead concentrations during childhood were excluded from the study. Adolescents were recruited in proportion to the racial and ethnic composition of the patient population (66.1% African American, 33.9% Caucasian, and 24.1% Hispanic). Informed consent was obtained from those that were willing to participate, and the study was approved by the Institutional Review Boards at the University of Rochester and Cornell University. Results on bone turnover and osteoprotegerin (151), maternal calcitropic hormones (67), fetal bone growth (152), maternal bone quality across gestation (153), placental vitamin D metabolic enzyme expression (76), placental VDR expression (71), expression of placental iron transporters (154, 155), maternal dietary intake (11), and maternal and neonatal iron status (156, 157) in this cohort have been published.

At entry into the study, important health and demographic data were obtained using a health survey demographic questionnaire. Self-reported information on maternal race, ethnicity, age, pre-pregnancy weight, socioeconomic status etc. was collected by the health project coordinator (**Appendix 1**). During the study, each adolescent attended up to three prenatal visits at early, mid, and late gestation. At each visit, maternal anthropometrics (height, weight, etc.) were recorded by clinical staff using standard procedures. A 24-hour dietary recall and prenatal

supplement survey were collected at each visit by study personnel. The recalls were analyzed by a registered dietician using the Nutrition Data System for Research (NDSR: University of Minnesota, Minneapolis, MN, versions 2006, 2008, and 2009) in Rochester, NY. Baby birth data was also collected (**Appendix 2**).

As part of the retrospective study, 25 pre-defined infections and inflammatory conditions were abstracted from their prenatal medical records by the health project coordinator using a study tool (**Appendix 3**). Archived blood samples collected during pregnancy (~26 weeks gestation), at delivery, and in the neonate at birth were used to undertake new analyses of inflammatory biomarkers. In addition, archived placental tissue samples were used to conduct new studies on placental vitamin D protein and placental antimicrobial peptide expression (**Figure 1.6**). Informed written consent was obtained from all participants and in participants 14 y of age and younger, both adolescent assent and parental consent were obtained (**Appendix 4**). The study was approved by the Institutional Review Boards at Cornell University and the University of Rochester.

Figure 1.6: Parent Study Timeline



REFERENCES

1. Bearinger LH, Sieving RE, Ferguson J, Sharma V. Global perspectives on the sexual and reproductive health of adolescents: patterns, prevention, and potential. *Lancet* 2007;369(9568):1220-31.
2. Kann L, Kinchen S, Shanklin SL, Flint KH, Kawkins J, Harris WA, et al. Youth risk behavior surveillance--United States, 2013. *MMWR Surveill Summ* 2014;63 Suppl 4:1-168.
3. Ford K, Sohn W, Lepkowski J. Characteristics of adolescents' sexual partners and their association with use of condoms and other contraceptive methods. *Fam Plann Perspect* 2001;33(3):100-5, 132.
4. Substance Abuse and Mental Health Services Administration, Results from the 2013 National Survey on Drug Use and Health: Summary of National Findings, NSDUH Series H-48, HHS Publication No. (SMA) 14-4863. Rockville, MD; 2014.
5. Tapert SF, Aarons GA, Sedlar GR, Brown SA. Adolescent substance use and sexual risk-taking behavior. *J Adolesc Health* 2001;28(3):181-9.
6. Hamilton BE MJ, Osterman MJK, Curtin SC. Preliminary data for 2014. National vital statistics reports; vol 64 no 6. Hyattsville, MD: National Center for Health Statistics. Available from: http://www.cdc.gov/nchs/data/nvsr/nvsr64/nvsr64_06.pdf. 2015. Accessed October 28 2016.
7. Sedgh G, Finer LB, Bankole A, Eilers MA, Singh S. Adolescent Pregnancy, Birth, and Abortion Rates Across Countries: Levels and Recent Trends. *J Adolesc Health* 2015;56(2):223-30.
8. Martin JA, Hamilton BE, Ventura SJ, Osterman MJ, Mathews TJ. Births: final data for 2011. *Natl Vital Stat Rep* 2013;62(1):1-69, 72.

9. Chen XK, Wen SW, Fleming N, Demissie K, Rhoads GG, Walker M. Teenage pregnancy and adverse birth outcomes: a large population based retrospective cohort study. *Int J Epidemiol* 2007;36(2):368-73.
10. Moran VH. A systematic review of dietary assessments of pregnant adolescents in industrialised countries. *Br J Nutr* 2007;97(3):411-25.
11. Lee S, Young BE, Cooper E, Pressman E, Queenan RA, Olson C, Guillet R, O'Brien KO. Nutrient inadequacy is prevalent in pregnant adolescents and prenatal supplement use may not fully compensate for dietary deficiencies. *Infant, Child, & Adolescent Nutrition* 2014;6:152-159.
12. Field CJ, Johnson IR, Schley PD. Nutrients and their role in host resistance to infection. *Journal of Leukocyte Biology* 2002;71(1):16-32.
13. Agrawal V, Hirsch E. Intrauterine infection and preterm labor. *Semin Fetal Neonatal Med* 2012;17(1):12-9.
14. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. *N Engl J Med* 2000;342(20):1500-7.
15. Tita ATN, Andrews WW. Diagnosis and Management of Clinical Chorioamnionitis. *Clinics in Perinatology* 2010;37(2):339-+.
16. Lamont RF. Infection in the prediction and antibiotics in the prevention of spontaneous preterm labour and preterm birth. *BJOG* 2003;110 Suppl 20:71-5.
17. Martin JA, Hamilton BE, Osterman MJ, Curtin SC, Matthews TJ. Births: final data for 2012. *Natl Vital Stat Rep* 2013;62(9):1-68.
18. Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, et al. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet* 2012;379(9832):2162-72.

19. Matuszkiewicz-Rowinska J, Malyszko J, Wieliczko M. Urinary tract infections in pregnancy: old and new unresolved diagnostic and therapeutic problems. *Arch Med Sci* 2015;11(1):67-77.
20. Matuszkiewicz-Rowinska J, Malyszko J, Wieliczko M. Urinary tract infections in pregnancy: old and new unresolved diagnostic and therapeutic problems. *Arch Med Sci* 2015;11(1):67-77.
21. van Schalkwyk J, Yudin MH, Infectious Disease C, Yudin MH, Allen V, Bouchard C, et al. Vulvovaginitis: screening for and management of trichomoniasis, vulvovaginal candidiasis, and bacterial vaginosis. *J Obstet Gynaecol Can* 2015;37(3):266-76.
22. Hillier SL, Nugent RP, Eschenbach DA, Krohn MA, Gibbs RS, Martin DH, et al. Association between bacterial vaginosis and preterm delivery of a low-birth-weight infant. The Vaginal Infections and Prematurity Study Group. *N Engl J Med* 1995;333(26):1737-42.
23. Nejad VM, Shafaie S. The association of bacterial vaginosis and preterm labor. *J Pak Med Assoc* 2008;58(3):104-6.
24. CDC. STDs during Pregnancy - CDC Fact Sheet 2016.
25. Talge NM, Holzman C, Wang J, Lucia V, Gardiner J, Breslau N. Late-preterm birth and its association with cognitive and socioemotional outcomes at 6 years of age. *Pediatrics* 2010;126(6):1124-31.
26. Kourtis AP, Read JS, Jamieson DJ. Pregnancy and Infection. *N Engl J Med* 2014;370(23):2211-2218.
27. Norman AW. Sunlight, season, skin pigmentation, vitamin D, and 25-hydroxyvitamin D: integral components of the vitamin D endocrine system. *Am J Clin Nutr* 1998;67(6):1108-10.
28. Duncan DD, Munoz B, Bandeen-Roche K, West SK. Assessment of ocular exposure to ultraviolet-B for population studies. Salisbury Eye Evaluation Project Team. *Photochem Photobiol* 1997;66(5):701-9.

29. Institute of Medicine. Dietary Reference Intakes for Calcium and Vitamin D. National Academy of Sciences Press. Washington (DC); 2011.
30. Calvo MS, Whiting SJ, Barton CN. Vitamin D fortification in the United States and Canada: current status and data needs. *Am J Clin Nutr* 2004;80(6 Suppl):1710S-6S.
31. Haddad JG, Matsuoka LY, Hollis BW, Hu YZ, Wortsman J. Human plasma transport of vitamin D after its endogenous synthesis. *J Clin Invest* 1993;91(6):2552-5.
32. Holick MF. Vitamin D: photobiology, metabolism, and clinical applications In *Endocrinology*, 3rd Edition.; 1995.
33. Reboul E, Goncalves A, Comera C, Bott R, Nowicki M, Landrier JF, et al. Vitamin D intestinal absorption is not a simple passive diffusion: evidences for involvement of cholesterol transporters. *Mol Nutr Food Res* 2011;55(5):691-702.
34. Stipanuk M, Caudill, MA. Biochemical, Physiological & Molecular Aspects of Human Nutrition. 3rd ed.: Saunders/Elsevier; 2012.
35. Jones G, Strugnell SA, DeLuca HF. Current understanding of the molecular actions of vitamin D. *Physiol Rev* 1998;78(4):1193-231.
36. Jones G. Pharmacokinetics of vitamin D toxicity. *Am J Clin Nutr* 2008;88(2):582S-586S.
37. Nykjaer A, Dragun D, Walther D, Vorum H, Jacobsen C, Herz J, et al. An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. *Cell* 1999;96(4):507-15.
38. Nykjaer A, Fyfe JC, Kozyraki R, Leheste JR, Jacobsen C, Nielsen MS, et al. Cubilin dysfunction causes abnormal metabolism of the steroid hormone 25(OH) vitamin D(3). *Proc Natl Acad Sci U S A* 2001;98(24):13895-900.
39. Judd SE, Tangpricha V. Vitamin D deficiency and risk for cardiovascular disease. *Am J Med Sci* 2009;338(1):40-4.
40. Hosking DJ. Calcium homeostasis in pregnancy. *Clinical Endocrinology* 1996;45(1):1-6.
41. Hewison M. Vitamin D and immune function: an overview. *Proc Nutr Soc* 2012;71(1):50-61.

42. White KE, Evans WE, O'Riordan JLH, Speer MC, Econs MJ, Lorenz-Depiereux B, et al. Autosomal dominant hypophosphataemic rickets is associated with mutations in FGF23. *Nature Genetics* 2000;26(3):345-348.
43. Liu SG, Tang W, Zhou JP, Stubbs JR, Luo Q, Pi M, et al. Fibroblast growth factor 23 is a counter-regulatory phosphaturic hormone for vitamin D. *J Am Soc Nephrol* 2006;17(5):1305-1315.
44. Rodriguez-Ortiz ME, Lopez I, Munoz-Castaneda JR, Martinez-Moreno JM, Ramirez AP, Pineda C, et al. Calcium deficiency reduces circulating levels of FGF23. *J Am Soc Nephrol* 2012;23(7):1190-7.
45. Dusso AS, Rodriguez M. Enhanced induction of Cyp24a1 by FGF23 but low serum 24,25-dihydroxyvitamin D in CKD: implications for therapy. *Kidney Int* 2012;82(10):1046-9.
46. Bai X, Miao D, Xiao S, Qiu D, St-Arnaud R, Petkovich M, et al. CYP24 inhibition as a therapeutic target in FGF23-mediated renal phosphate wasting disorders. *J Clin Invest* 2016;126(2):667-80.
47. Torres PA, De Brauwere DP. Three feedback loops precisely regulating serum phosphate concentration. *Kidney Int* 2011;80(5):443-5.
48. Bouillon R, Carmeliet G, Verlinden L, van Etten E, Verstuyf A, Luderer HF, et al. Vitamin D and human health: lessons learned from vitamin D receptor null mice. *Endocr Rev* 2008;29(6):726-76.
49. Turunen MM, Dunlop TW, Carlberg C, Vaisanen S. Selective use of multiple vitamin D response elements underlies the 1 alpha,25-dihydroxyvitamin D3-mediated negative regulation of the human CYP27B1 gene. *Nucleic Acids Res* 2007;35(8):2734-47.
50. Aslan S, Akil I, Aslan G, Onay H, Ozyurt BC, Ozkinay F. Vitamin D receptor gene polymorphism in children with urinary tract infection. *Pediatr Nephrol* 2012;27(3):417-21.

51. Han WG, Hodemaekers HM, Nagarajah B, Poelen MM, Helm K, Janssen R, et al. Association of Vitamin D Receptor Polymorphism with Susceptibility to Symptomatic Pertussis. *PLoS One* 2016;11(2):e0149576.
52. Holick CN, Stanford JL, Kwon EM, Ostrander EA, Nejentsev S, Peters U. Comprehensive association analysis of the vitamin D pathway genes, VDR, CYP27B1, and CYP24A1, in prostate cancer. *Cancer Epidemiol Biomarkers Prev* 2007;16(10):1990-9.
53. Zeljic K, Supic G, Stamenkovic Radak M, Jovic N, Kozomara R, Magic Z. Vitamin D receptor, CYP27B1 and CYP24A1 genes polymorphisms association with oral cancer risk and survival. *J Oral Pathol Med* 2012;41(10):779-87.
54. Powe CE, Karumanchi SA, Thadhani R. Vitamin D-Binding Protein and Vitamin D in Blacks and Whites. *New England Journal of Medicine* 2013;369:1999-2000.
55. Zhang YL, Yang SF, Liu Y, Ren LH. Relationship between polymorphisms in vitamin D metabolism-related genes and the risk of rickets in Han Chinese children. *BMC Medical Genetics* 2013;14.
56. Kuan V, Martineau AR, Griffiths CJ, Hypponen E, Walton R. DHCR7 mutations linked to higher vitamin D status allowed early human migration to Northern latitudes. *BMC Evolutionary Biology* 2013;13.
57. Walker VP, Modlin RL. The Vitamin D Connection to Pediatric Infections and Immune Function. *Pediatric Research* 2009;65(5):106r-113r.
58. Laaksi I, Ruohola JP, Tuohimaa P, Auvinen A, Haataja R, Pihlajamaki H, et al. An association of serum vitamin D concentrations < 40 nmol/L with acute respiratory tract infection in young Finnish men. *Am J Clin Nutr* 2007;86(3):714-7.
59. Nseir W, Taha M, Nemarny H, Mograbi J. The association between serum levels of vitamin D and recurrent urinary tract infections in premenopausal women. *International Journal of Infectious Diseases* 2013;17(12):E1121-E1124.

60. Merlino LA, Curtis J, Mikuls TR, Cerhan JR, Criswell LA, Saag KG. Vitamin D intake is inversely associated with rheumatoid arthritis: results from the Iowa Women's Health Study. *Arthritis Rheum* 2004;50(1):72-7.
61. Hypponen E, Laara E, Reunanen A, Jarvelin MR, Virtanen SM. Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. *Lancet* 2001;358(9292):1500-3.
62. Garland CF, Comstock GW, Garland FC, Helsing KJ, Shaw EK, Gorham ED. Serum 25-hydroxyvitamin D and colon cancer: eight-year prospective study. *Lancet* 1989;2(8673):1176-8.
63. Hayes CE. Vitamin D: a natural inhibitor of multiple sclerosis. *Proc Nutr Soc* 2000;59(4):531-5.
64. Kamen DL, Cooper GS, Bouali H, Shaftman SR, Hollis BW, Gilkeson GS. Vitamin D deficiency in systemic lupus erythematosus. *Autoimmun Rev* 2006;5(2):114-7.
65. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J Clin Endocrinol Metab* 2011;96(1):53-8.
66. Kovacs CS. The role of vitamin D in pregnancy and lactation: insights from animal models and clinical studies. *Annu Rev Nutr* 2012;32:97-123.
67. Young BE, McNanley TJ, Cooper EM, McIntyre AW, Witter F, Harris ZL, et al. Vitamin D insufficiency is prevalent and vitamin D is inversely associated with parathyroid hormone and calcitriol in pregnant adolescents. *J Bone Miner Res* 2012;27(1):177-86.
68. Hollis BW, Johnson D, Hulsey TC, Ebeling M, Wagner CL. Vitamin D supplementation during pregnancy: double-blind, randomized clinical trial of safety and effectiveness. *J Bone Miner Res* 2011;26(10):2341-57.
69. Mahon P, Harvey N, Crozier S, Inskip H, Robinson S, Arden N, et al. Low maternal vitamin D status and fetal bone development: cohort study. *J Bone Miner Res* 2010;25(1):14-9.

70. Young BE, McNanley TJ, Cooper EM, McIntyre AW, Witter F, Harris ZL, et al. Maternal vitamin D status and calcium intake interact to affect fetal skeletal growth in utero in pregnant adolescents. *American Journal of Clinical Nutrition* 2012;95(5):1103-12.
71. Young BE, Cooper EM, McIntyre AW, Kent T, Witter F, Harris ZL, et al. Placental vitamin D receptor (VDR) expression is related to neonatal vitamin D status, placental calcium transfer, and fetal bone length in pregnant adolescents. *FASEB J* 2014;28(5):2029-37.
72. Wjst M. Is vitamin D supplementation responsible for the allergy pandemic? *Curr Opin Allergy Clin Immunol* 2012;12(3):257-62.
73. Reichetzeder C, Chen H, Foller M, Slowinski T, Li J, Chen YP, et al. Maternal Vitamin D Deficiency and Fetal Programming - Lessons Learned from Humans and Mice. *Kidney & Blood Pressure Research* 2014;39(4):315-329.
74. Delvin EE, Glorieux FH, Salle BL, David L, Varenne JP. Control of vitamin D metabolism in preterm infants: feto-maternal relationships. *Arch Dis Child* 1982;57(10):754-7.
75. Gertner JM, Glassman MS, Coustan DR, Goodman DB. Fetomaternal vitamin D relationships at term. *J Pediatr* 1980;97(4):637-40.
76. O'Brien KO, Li S, Cao C, Kent T, Young BV, Queenan RA, et al. Placental CYP27B1 and CYP24A1 expression in human placental tissue and their association with maternal and neonatal calcitropic hormones. *J Clin Endocrinol Metab* 2014;99(4):1348-56.
77. Hollis BW, Pittard WB, 3rd. Evaluation of the total fetomaternal vitamin D relationships at term: evidence for racial differences. *J Clin Endocrinol Metab* 1984;59(4):652-7.
78. Burke KA, Jauniaux E, Burton GJ, Cindrova-Davies T. Expression and immunolocalisation of the endocytic receptors megalin and cubilin in the human yolk sac and placenta across gestation. *Placenta* 2013;34(11):1105-9.

79. Ritchie LD, Fung EB, Halloran BP, Turnlund JR, Van Loan MD, Cann CE, et al. A longitudinal study of calcium homeostasis during human pregnancy and lactation and after resumption of menses. *American Journal of Clinical Nutrition* 1998;67(4):693-701.
80. Kovacs CS. Calcium and bone metabolism during pregnancy and lactation. *Journal of Mammary Gland Biology and Neoplasia* 2005;10(2):105-118.
81. Gray TK, Lester GE, Lorenc RS. Evidence for extra-renal 1 alpha-hydroxylation of 25-hydroxyvitamin D3 in pregnancy. *Science* 1979;204(4399):1311-3.
82. Weisman Y, Harell A, Edelstein S, David M, Spirer Z, Golander A. 1 alpha, 25-Dihydroxyvitamin D3 and 24,25-dihydroxyvitamin D3 in vitro synthesis by human decidua and placenta. *Nature* 1979;281(5729):317-9.
83. Barrera D, Avila E, Hernandez G, Halhali A, Biruete B, Larrea F, et al. Estradiol and progesterone synthesis in human placenta is stimulated by calcitriol. *Journal of Steroid Biochemistry and Molecular Biology* 2007;103(3-5):529-532.
84. Stephanou A, Ross R, Handwerger S. Regulation of Human Placental-Lactogen Expression by 1,25-Dihydroxyvitamin D-3. *Endocrinology* 1994;135(6):2651-2656.
85. Barrera D, Avila E, Hernandez G, Mendez I, Gonzalez L, Halhali A, et al. Calcitriol affects hCG gene transcription in cultured human syncytiotrophoblasts. *Reproductive Biology and Endocrinology* 2008;6.
86. Novakovic B, Sibson M, Ng HK, Manuelpillai U, Rakyan V, Down T, et al. Placenta-specific methylation of the vitamin D 24-hydroxylase gene: implications for feedback autoregulation of active vitamin D levels at the fetomaternal interface. *J Biol Chem* 2009;284(22):14838-48.
87. Noyola-Martinez N, Diaz L, Avila E, Halhali A, Larrea F, Barrera D. Calcitriol downregulates TNF-alpha and IL-6 expression in cultured placental cells from preeclamptic women. *Cytokine* 2013;61(1):245-50.
88. Liu NQ, Hewison M. Vitamin D, the placenta and pregnancy. *Arch Biochem Biophys* 2012;523(1):37-47.

89. Bodnar LM, Catov JM, Simhan HN, Holick MF, Powers RW, Roberts JM. Maternal vitamin D deficiency increases the risk of preeclampsia. *J Clin Endocrinol Metab* 2007;92(9):3517-22.
90. Bodnar LM, Krohn MA, Simhan HN. Maternal vitamin D deficiency is associated with bacterial vaginosis in the first trimester of pregnancy. *J Nutr* 2009;139(6):1157-61.
91. Merewood A, Mehta SD, Chen TC, Bauchner H, Holick MF. Association between vitamin D deficiency and primary cesarean section. *J Clin Endocrinol Metab* 2009;94(3):940-5.
92. Bodnar LM, Catov JM, Zmuda JM, Cooper ME, Parrott MS, Roberts JM, et al. Maternal serum 25-hydroxyvitamin D concentrations are associated with small-for-gestational age births in white women. *J Nutr* 2010;140(5):999-1006.
93. Wagner CL, McNeil RB, Johnson DD, Hulsey TC, Ebeling M, Robinson C, et al. Health characteristics and outcomes of two randomized vitamin D supplementation trials during pregnancy: a combined analysis. *J Steroid Biochem Mol Biol* 2013;136:313-20.
94. Ginde AA, Sullivan AF, Mansbach JM, Camargo CA, Jr. Vitamin D insufficiency in pregnant and nonpregnant women of childbearing age in the United States. *American Journal of Obstetrics and Gynecology* 2010;202(5):436 e1-8.
95. Weinstock H, Berman S, Cates W, Jr. Sexually transmitted diseases among American youth: incidence and prevalence estimates, 2000. *Perspect Sex Reprod Health* 2004;36(1):6-10.
96. Bodnar LM, Simhan HN, Catov JM, Roberts JM, Platt RW, Diesel JC, et al. Maternal vitamin D status and the risk of mild and severe preeclampsia. *Epidemiology* 2014;25(2):207-14.
97. Naghshineh E, Sheikhalian S. Effect of vitamin D supplementation in the reduce risk of preeclampsia in nulliparous women. *Adv Biomed Res* 2016;5:7.
98. Bodnar LM, Platt RW, Simhan HN. Early-pregnancy vitamin D deficiency and risk of preterm birth subtypes. *Obstet Gynecol* 2015;125(2):439-47.

99. Wagner CL, Baggerly C, McDonnell S, Baggerly KA, French CB, Baggerly L, et al. Post-hoc analysis of vitamin D status and reduced risk of preterm birth in two vitamin D pregnancy cohorts compared with South Carolina March of Dimes 2009-2011 rates. *J Steroid Biochem Mol Biol* 2016;155(Pt B):245-51.
100. Wagner CL, McNeil R, Hamilton SA, Winkler J, Rodriguez Cook C, Warner G, et al. A randomized trial of vitamin D supplementation in 2 community health center networks in South Carolina. *Am J Obstet Gynecol* 2013;208(2):137 e1-13.
101. Burris HH, Rifas-Shiman SL, Kleinman K, Litonjua AA, Huh SY, Rich-Edwards JW, et al. Vitamin D deficiency in pregnancy and gestational diabetes mellitus. *Am J Obstet Gynecol* 2012;207(3).
102. Clifton-Bligh RJ, McElduff P, McElduff A. Maternal vitamin D deficiency, ethnicity and gestational diabetes. *Diabetic Medicine* 2008;25(6):678-684.
103. Farrant HJW, Krishnaveni GV, Hill JC, Boucher BJ, Fisher DJ, Noonan K, et al. Vitamin D insufficiency is common in Indian mothers but is not associated with gestational diabetes or variation in newborn size. *European Journal of Clinical Nutrition* 2009;63(5):646-652.
104. Makgoba M, Nelson SM, Savvidou M, Messow CM, Nicolaides K, Sattar N. First-trimester circulating 25-hydroxyvitamin D levels and development of gestational diabetes mellitus. *Diabetes Care* 2011;34(5):1091-3.
105. Gernand AD, Klebanoff MA, Simhan HN, Bodnar LM. Maternal vitamin D status, prolonged labor, cesarean delivery and instrumental delivery in an era with a low cesarean rate. *J Perinatol* 2015;35(1):23-8.
106. Scholl TO, Chen X, Stein P. Maternal vitamin D status and delivery by cesarean. *Nutrients* 2012;4(4):319-30.
107. Abbas AK L, AH, Pillai S. Cellular and Molecular Immunology. Elsevier 2012;7th ed.
108. Bikle DD. Vitamin D: newly discovered actions require reconsideration of physiologic requirements. *Trends in Endocrinology and Metabolism* 2010;21(6):375-384.

109. Lehrer RI, Ganz T. Antimicrobial peptides in mammalian and insect host defence. *Curr Opin Immunol* 1999;11(1):23-7.
110. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* 2006;311(5768):1770-3.
111. Adams JS, Ren S, Liu PT, Chun RF, Lagishetty V, Gombart AF, et al. Vitamin d-directed rheostatic regulation of monocyte antibacterial responses. *J Immunol* 2009;182(7):4289-95.
112. Wang TT, Nestel FP, Bourdeau W, Nagai Y, Wang QY, Liao J, et al. Cutting edge: 1,25-dihydroxyvitamin D-3 is a direct inducer of antimicrobial peptide gene expression. *J Immunol* 2004;173(5):2909-2912.
113. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, et al. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J Immunol* 2004;173(5):2909-12.
114. Thoma-Uszynski S, Stenger S, Takeuchi O, Ochoa MT, Engele M, Sieling PA, et al. Induction of direct antimicrobial activity through mammalian toll-like receptors. *Science* 2001;291(5508):1544-7.
115. Grundmann V-H, FV. Vitamin D-roles in women's reproductive health? *Reproductive Biology and Endocrinology* 2011;9:146.
116. King AE, Paltoo A, Kelly RW, Sallenave JM, Bocking AD, Challis JR. Expression of natural antimicrobials by human placenta and fetal membranes. *Placenta* 2007;28(2-3):161-9.
117. Liu N, Kaplan AT, Low J, Nguyen L, Liu GY, Equils O, et al. Vitamin D induces innate antibacterial responses in human trophoblasts via an intracrine pathway. *Biol Reprod* 2009;80(3):398-406.
118. Bacchetta J, Zaritsky JJ, Sea JL, Chun RF, Lisse TS, Zavala K, et al. Suppression of iron-regulatory hepcidin by vitamin D. *J Am Soc Nephrol* 2014;25(3):564-72.

119. Collins HL. The role of iron in infections with intracellular bacteria. *Immunol Lett* 2003;85(2):193-5.
120. Drakesmith H, Prentice AM. Hepcidin and the iron-infection axis. *Science* 2012;338(6108):768-72.
121. Lysenko ES, Gould J, Bals R, Wilson JM, Weiser JN. Bacterial phosphorylcholine decreases susceptibility to the antimicrobial peptide LL-37/hCAP18 expressed in the upper respiratory tract. *Infect Immun* 2000;68(3):1664-71.
122. Mangin M, Sinha R, Fincher K. Inflammation and vitamin D: the infection connection. *Inflamm Res* 2014;63(10):803-19.
123. Jeffery LE, Burke F, Mura M, Zheng Y, Qureshi OS, Hewison M, et al. 1,25-Dihydroxyvitamin D-3 and IL-2 Combine to Inhibit T Cell Production of Inflammatory Cytokines and Promote Development of Regulatory T Cells Expressing CTLA-4 and FoxP3. *J Immunol* 2009;183(9):5458-5467.
124. Rostkowska-Nadolska B, Sliupkas-Dyrda E, Potyka J, Kusmierz D, Fraczek M, Krecicki T, et al. Vitamin D derivatives: calcitriol and tacalcitol inhibits interleukin-6 and interleukin-8 expression in human nasal polyp fibroblast cultures. *Adv Med Sci* 2010;55(1):86-92.
125. Heine G, Niesner U, Chang HD, Steinmeyer A, Zugel U, Zuberbier T, et al. 1,25-dihydroxyvitamin D-3 promotes IL-10 production in human B cells. *European Journal of Immunology* 2008;38(8):2210-2218.
126. Michel G, Gailis A, Jarzebska-Deussen B, Muschen A, Mirmohammadsadegh A, Ruzicka T. 1,25-(OH)₂-vitamin D₃ and calcipotriol induce IL-10 receptor gene expression in human epidermal cells. *Inflamm Res* 1997;46(1):32-4.
127. Brennan A, Katz DR, Nunn JD, Barker S, Hewison M, Fraher LJ, et al. Dendritic cells from human tissues express receptors for the immunoregulatory vitamin D₃ metabolite, dihydroxycholecalciferol. *Immunology* 1987;61(4):457-61.

128. Hewison M, Freeman L, Hughes SV, Evans KN, Bland R, Eliopoulos AG, et al. Differential regulation of vitamin D receptor and its ligand in human monocyte-derived dendritic cells. *J Immunol* 2003;170(11):5382-90.
129. Adorini L. Tolerogenic dendritic cells induced by vitamin D receptor ligands enhance regulatory T cells inhibiting autoimmune diabetes. *Ann N Y Acad Sci* 2003;987:258-61.
130. Penna G, Adorini L. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J Immunol* 2000;164(5):2405-11.
131. Griffin MD, Lutz W, Phan VA, Bachman LA, McKean DJ, Kumar R. Dendritic cell modulation by 1alpha,25 dihydroxyvitamin D3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo. *Proc Natl Acad Sci U S A* 2001;98(12):6800-5.
132. Panda DK, Miao D, Tremblay ML, Sirois J, Farookhi R, Hendy GN, et al. Targeted ablation of the 25-hydroxyvitamin D 1alpha -hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. *Proc Natl Acad Sci U S A* 2001;98(13):7498-503.
133. Bhalla AK, Amento EP, Clemens TL, Holick MF, Krane SM. Specific high-affinity receptors for 1,25-dihydroxyvitamin D3 in human peripheral blood mononuclear cells: presence in monocytes and induction in T lymphocytes following activation. *J Clin Endocrinol Metab* 1983;57(6):1308-10.
134. Provvedini DM, Tsoukas CD, Deftos LJ, Manolagas SC. 1,25-dihydroxyvitamin D3 receptors in human leukocytes. *Science* 1983;221(4616):1181-3.
135. Lemire JM, Archer DC, Beck L, Spiegelberg HL. Immunosuppressive actions of 1,25-dihydroxyvitamin D3: preferential inhibition of Th1 functions. *J Nutr* 1995;125(6 Suppl):1704S-1708S.

136. Overbergh L, Decallonne B, Waer M, Rutgeerts O, Valckx D, Casteels KM, et al. 1alpha,25-dihydroxyvitamin D3 induces an autoantigen-specific T-helper 1/T-helper 2 immune shift in NOD mice immunized with GAD65 (p524-543). *Diabetes* 2000;49(8):1301-7.
137. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A. 1alpha,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. *J Immunol* 2001;167(9):4974-80.
138. Liu N, Nguyen L, Chun RF, Lagishetty V, Ren S, Wu S, et al. Altered endocrine and autocrine metabolism of vitamin D in a mouse model of gastrointestinal inflammation. *Endocrinology* 2008;149(10):4799-808.
139. Bettelli E, Korn T, Kuchroo VK. Th17: the third member of the effector T cell trilogy. *Curr Opin Immunol* 2007;19(6):652-7.
140. Korn T, Oukka M, Kuchroo V, Bettelli E. Th17 cells: effector T cells with inflammatory properties. *Semin Immunol* 2007;19(6):362-71.
141. Colin EM, Asmawidjaja PS, van Hamburg JP, Mus AM, van Driel M, Hazes JM, et al. 1,25-dihydroxyvitamin D3 modulates Th17 polarization and interleukin-22 expression by memory T cells from patients with early rheumatoid arthritis. *Arthritis Rheum* 2010;62(1):132-42.
142. Palmer MT, Lee YK, Maynard CL, Oliver JR, Bikle DD, Jetten AM, et al. Lineage-specific effects of 1,25-dihydroxyvitamin D(3) on the development of effector CD4 T cells. *J Biol Chem* 2011;286(2):997-1004.
143. Gorman S, Kuritzky LA, Judge MA, Dixon KM, McGlade JP, Mason RS, et al. Topically applied 1,25-dihydroxyvitamin D3 enhances the suppressive activity of CD4+CD25+ cells in the draining lymph nodes. *J Immunol* 2007;179(9):6273-83.

144. Jeffery LE, Burke F, Mura M, Zheng Y, Qureshi OS, Hewison M, et al. 1,25-Dihydroxyvitamin D3 and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3. *J Immunol* 2009;183(9):5458-67.
145. Urry Z, Xystrakis E, Richards DF, McDonald J, Sattar Z, Cousins DJ, et al. Ligation of TLR9 induced on human IL-10-secreting Tregs by 1alpha,25-dihydroxyvitamin D3 abrogates regulatory function. *J Clin Invest* 2009;119(2):387-98.
146. Chen S, Sims GP, Chen XX, Gu YY, Chen S, Lipsky PE. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. *J Immunol* 2007;179(3):1634-47.
147. Zerofsky MS, Jacoby BN, Pedersen TL, Stephensen CB. Daily Cholecalciferol Supplementation during Pregnancy Alters Markers of Regulatory Immunity, Inflammation, and Clinical Outcomes in a Randomized Controlled Trial. *J Nutr* 2016;146(11):2388-2397.
148. Barrera D, Noyola-Martinez N, Avila E, Halhali A, Larrea F, Diaz L. Calcitriol inhibits interleukin-10 expression in cultured human trophoblasts under normal and inflammatory conditions. *Cytokine* 2012;57(3):316-21.
149. Saito S, Sakai M. Th1/Th2 balance in preeclampsia. *J Reprod Immunol* 2003;59(2):161-73.
150. Romero R, Erez O, Espinoza J. Intrauterine infection, preterm labor, and cytokines. *J Soc Gynecol Investig* 2005;12(7):463-5.
151. Essley B, McNanley T, Cooper B, McIntyre A, Witter F, Harris Z, et al. Osteoprotegerin in pregnant adolescents differs by race and is related to infant birth weight z-score. *J Dev Orig Health Dis* 2011;2(5):272-9.
152. Young BE, McNanley TJ, Cooper EM, McIntyre AW, Witter F, Harris ZL, et al. Maternal vitamin D status and calcium intake interact to affect fetal skeletal growth in utero in pregnant adolescents. *Am J Clin Nutr* 2012;95(5):1103-12.

153. Whisner CM, Young BE, Witter FR, Harris ZL, Queenan RA, Cooper EM, et al. Reductions in Heel Bone Quality Across Gestation Are Attenuated in Pregnant Adolescents With Higher Prepregnancy Weight and Greater Increases in PTH Across Gestation. *J Bone Miner Res* 2014;29(9):2109-17.
154. Young MF, Pressman E, Foehr ML, McNanley T, Cooper E, Guillet R, et al. Impact of maternal and neonatal iron status on placental transferrin receptor expression in pregnant adolescents. *Placenta* 2010;31(11):1010-4.
155. Jaacks LM, Young MF, Essley BV, McNanley TJ, Cooper EM, Pressman EK, et al. Placental expression of the heme transporter, feline leukemia virus subgroup C receptor, is related to maternal iron status in pregnant adolescents. *J Nutr* 2011;141(7):1267-72.
156. Lee S, Guillet R, Cooper EM, Westerman M, Orlando M, Pressman E, et al. Maternal inflammation at delivery affects assessment of maternal iron status. *J Nutr* 2014;144(10):1524-32.
157. Lee S, Guillet R, Cooper EM, Westerman M, Orlando M, Kent T, et al. Prevalence of anemia and associations between neonatal iron status, hepcidin, and maternal iron status among neonates born to pregnant adolescents. *Pediatr Res* 2016;79(1-1):42-8.

CHAPTER 2

Prevalence and Risk Factors for Infections in a Pregnant Adolescent Population

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ABSTRACT

Study Objective

Our objective was to identify risk factors associated with maternal infections and placental inflammation in pregnant adolescents attending an urban adolescent maternity clinic.

Design

This cross-sectional, descriptive study used survey and medical chart data collected at entry and prospectively across gestation. The prevalence of maternal infections and placental inflammation was determined and potential risk factors were identified.

Setting

Rochester Adolescent Maternal Program (RAMP) in Rochester, NY

Participants

Racially and ethnically diverse pregnant adolescents ($n=158 \leq 18$ y at entry) were recruited.

Main Outcome Measures

Main outcome measures were diagnosis of an infection or inflammatory condition in relation to demographic, anthropometric, dietary, socioeconomic, and health data.

Results

The three most prevalent infections diagnosed in this study population were recto-vaginal colonization of group B Streptococcus (GBS) (38%), bacterial vaginosis (BV) (40%) and candida (42%). African-American teens (AOR=4.6; 95% CI: 1.74-13.02) and those with higher pre-pregnancy BMI (ppBMI; AOR=1.2; 95% CI: 1.04-1.31) were more likely to test positive for BV across gestation. Older maternal age decreased the likelihood of positive tests for trichomoniasis (OR=0.51; 95% CI: 0.26-0.92) and gonorrhea (OR=0.38; 95% CI: 0.16-0.82). Higher mean dietary vitamin D intake (mcg/d) was associated with a lower likelihood of testing

positive for recto-vaginal GBS (OR=0.87; 95% CI: 0.77-0.98).

Conclusions

Addressing modifiable risk factors associated with dietary intake and pre-pregnancy weight may help reduce health disparities among pregnant minority adolescents. Additionally, targeted sexual health education may greatly benefit younger female adolescents.

INTRODUCTION

The U.S. teen pregnancy rate remains the highest among developed countries (57 pregnancies per 1,000 females ages 15-19 years)(1) with Hispanic and African-American teens comprising the majority of this population.(2) Unplanned pregnancies pose significant challenges to reproductive health as they have been linked to risky sexual behaviors(3) and adverse infant health outcomes.(4) Compared to young adult women (aged 20-24 years), pregnant teens are more likely to be unmarried, have inadequate prenatal care, and not achieve recommended pregnancy weight gains. In addition, neonates born to teens are at greater risk for low Apgar scores, prematurity, and increased risk of neonatal mortality.(5)

During pregnancy, sexually-transmitted, urogenital, and placental infections are of major concern. U.S. teens and young adults (15-24 years old) comprise 50% of new sexually-transmitted infection (STI) cases each year.(6) Maternal infections during pregnancy and their transmission to the neonate during labor and delivery have been linked to adverse health outcomes, including premature birth,(7) low birth weight (LBW) (<2500 g),(8) and newborn lung and eye infections.(9) Therefore, the Centers for Disease Control and Prevention (CDC) recommends screening all pregnant females for chlamydia, hepatitis B, human immunodeficiency virus (HIV), and syphilis over the course of pregnancy and those deemed

high-risk should also be screened for gonorrhea and hepatitis C.(10) In addition, screening for recto-vaginal group B streptococcal colonization (GBS) is recommended for all pregnant women between 35-37 weeks of gestation.(10) In contrast, some infections such as bacterial vaginosis (BV) are not routinely screened for during pregnancy despite a growing body of research linking BV to undesirable birth outcomes.(11)

Most of the research exploring infection risk factors has focused on adult populations and risk factors vary depending on the infection in question. Previously identified risk factors for BV include low socioeconomic status, race, smoking, higher body mass index (BMI), having a past pregnancy, and increased number of sexual partners.(12,13) Urinary tract infections are common during pregnancy, and the American College of Obstetrics and Gynecology (ACOG) recommends screening all pregnant women for asymptomatic bacteriuria during the first prenatal visit and third trimester.(14) Prior studies have reported several risk factors for urinary tract infections (UTIs) occurring over pregnancy, including pre-eclampsia, hypertension, low socioeconomic status, and anemia.(15) Younger age (16,17) and the consumption of fruit juices and milk products containing probiotics has also been associated with a reduced risk of recurrent UTIs in non-pregnant adult women.(18)

Much less is known about risk factors in other age groups and little is known about these relationships in pregnant adolescents, a population which is at increased risk for STIs and adverse neonatal outcomes. The primary objective of this study was to describe the prevalence of, and identify potential risk factors (health behaviors, demographic, socioeconomic, anthropometric, and dietary) for common infections across gestation in a racially and ethnically diverse group of pregnant adolescents (≤ 18 years of age).

MATERIALS AND METHODS

Study Population

A group of 158 pregnant adolescents (≤ 18 years old) were recruited from the Rochester Adolescent Maternity Program (RAMP) in Rochester, New York from 2006-2009 for a study designed to examine maternal and fetal bone health. Adolescents were recruited in proportion to the racial and ethnic composition of the patient population (65% African American, 35% Caucasian, and 25% Hispanic) (**Table 2.1**).

Table 2.1: Subject Characteristics of the 158 Pregnant Adolescents^a

Characteristics	Value
Age at enrollment (years)	17.1 \pm 1.1 (158)
Race, %	
African American	63.3 (100/158)
Caucasian	35.4 (56/158)
Native American	1.3 (2/158)
Ethnicity, %	
Hispanic	24.7 (39/158)
Non-Hispanic	75.3 (119/158)
GA at entry into prenatal care (wks)	10.5 \pm 4.7 (137)
GA at delivery (wks)	39.2 \pm 3.0 (152)
Pre-pregnancy BMI (ppBMI) (kg/m ²)	24.7 \pm 5.4 (156)
Underweight (<18.5), %	6.4 (10/156)
Normal (18.5-24.9), %	57 (89/156)
Overweight (≥ 25.0), %	19 (30/156)
Obese (≥ 30.0), %	17 (27/156)
WIC enrollment, %	72.6 (114/157)
Prior birth control use, %	16.5 (26/158)

^aValues are presented as the mean \pm SD or % (n); GA, Gestational age; WIC, Special Supplemental Nutrition Program for Women, Infants, and Children.

All eligible adolescents were approached by the study coordinator, and the majority agreed to volunteer to participate in this research study. Participants were eligible for the study if they were between 12 and 30 weeks gestation at entry, carrying a single fetus, and at least 13 years of age. Additional eligibility and exclusion criteria have been previously described.(19)

Adolescents were longitudinally followed from study entry until delivery and medical chart data were abstracted from entry into prenatal care until delivery. Informed written consent was obtained from all participants after fully explaining the study and study procedures in accordance with the institutional guidelines. The study was approved by the Institutional Review Boards at Cornell University and the University of Rochester.

Data Collection

At study entry, key health and demographic data were obtained by the study health project coordinator as previously described.(19, 20) Socioeconomic status indicators assessed included health insurance, participation in the Special Supplemental Nutrition Program for Women, Infants, and Children (WIC), or welfare/public assistance programs. Self-reported risk taking behaviors (before and during pregnancy) were queried, including use of alcohol and cigarettes categorized as never used, prior use, or current use. Self-reported use of marijuana as well as “other drugs” (cocaine, stimulants, sedatives, and narcotics) were described as ever used versus never used before or during pregnancy. Birth control use (yes/no) prior to conception was ascertained. Pre-pregnancy body mass index (ppBMI) was calculated and categorized as underweight ($<18.5 \text{ kg/m}^2$), normal weight ($18.5\text{-}24.9 \text{ kg/m}^2$), overweight ($\geq 25.0 \text{ kg/m}^2$), or obese ($\geq 30.0 \text{ kg/m}^2$) using self-reported height and weight following the World Health Organization guidelines.(21)

Adolescents attended up to three prenatal study visits, at which time maternal anthropometrics and all survey instruments were completed. The mean micronutrient and macronutrient intakes were obtained using a 24-hour dietary recall and the estimated average requirement (EAR) and acceptable macronutrient distribution range (AMDR) were used to classify adequate vs. inadequate nutrient intake as previously described.(20) A prenatal

supplement survey was used to assess frequency of supplement as daily vs. not daily for analysis purposes.

Data on 18 pre-defined infections and inflammatory conditions were abstracted from the participant's prenatal medical records (STIs, UTIs, vaginal infections, placental inflammatory conditions, and other viral and bacterial infections) by our study health project coordinator. The prevalence of each condition was calculated and presented in **Table 2.2**. For each condition reported, the date of clinician diagnosis or date of laboratory results for the condition and prescribed treatments were recorded. Classifications used for conditions monitored were determined by obstetrics and gynecology clinicians and co-authors at the University of Rochester Medical Center (URMC).

Table 2.2: Prevalence of Maternal Infection and Inflammatory Conditions Across Pregnancy in 158 Pregnant Adolescents

<u>Sexually Transmitted Infections (STIs):</u>		<u>Vaginal Infections:</u>	
Gonorrhea	3%	Candida	42%
Chlamydia	13%	Bacterial Vaginosis	40%
Syphilis	0.6%	<u>Placental Inflammation (n=45)^a:</u>	
Genital Warts	4%	Chorioamnionitis	14%
Genital Herpes	1%	Chronic Vilitis	0.6%
Trichomoniasis	7%	Deciduitis	9%
Human Papillomavirus	7.6%	Acute Funisitis	11%
		<u>Other Infections:</u>	
<u>Urinary Tract Infections (UTIs):</u>		Hepatitis B	0%
Pyelonephritis	1%	Hepatitis C	0.6%
Cystitis	25%	<u>Other (Carrier State):</u>	
		Group B Strep	38%

^aValues presented are only from a sub-sample of adolescents (N=45) whose placentas were sent to pathology for medically indicated purposes.

Statistical Analysis

Potential determinants of infections and inflammatory conditions including: demographic factors (race, ethnicity, maternal age at time of infection diagnosis, age at menarche, gynecological age at conception), socioeconomic status (SES) (WIC participation, use of public assistance, and health insurance participation), anthropometric factors (ppBMI), dietary factors (micronutrient and macronutrient intake), and health behaviors (self-reported use of prenatal supplements and substance abuse behaviors) were assessed using chi-square test of independence for categorical variables and bivariate logistic regression for continuous variables. Fisher's exact test was used when data in cross tabulations were sparse or unbalanced ($n < 5$). Outcomes of infection (positive/negative) and inflammation (presence/absence) were analyzed as categorical variables while potential risk factors were analyzed as either categorical or continuous variables. Multiple logistic regression was used to simultaneously assess the association of multiple risk factors on infection outcomes. Results were considered statistically significant at $P < 0.05$. All analyses were performed using JMP 11.0 (SAS Institute).

RESULTS

In this study population, 73% (114/157) of teens were enrolled in WIC, 18% (22/121) used public assistance programs, and 91% (143/158) had some form of health insurance. Most of the teens 68% (108/158) reported that they had obtained at least a 10th grade or higher level of education. From among the indicators of socioeconomic status available, none were significantly associated with the outcomes of interest.

Of the infections and inflammatory conditions presented in **Table 2.2**, 30.4% (48/158) of teens had at least one diagnosed STI, 25.3% (40/158) had one, 3.8% (6/158) had two, and 1.3%

(2/158) had three STIs across gestation. For the 64% (101/158) of teens found to have at least one vaginal infection, 45.6% (72/158) had one and 18.4% (29/158) had two recorded diagnoses across gestation. For teens that had their placentas sent to pathology at delivery due to medically indicated concerns, 60% (27/45) had pathologic evidence of placental inflammation. The odds of having a vaginal infection were 2.4 times higher in those with a STI (OR=2.4; 95% CI: 1.1-5.4) compared to teens who were not diagnosed with a STI during pregnancy.

Significant associations between study outcomes and maternal race, age at conception, and maternal age were noted. The unadjusted odds of having bacterial vaginosis were 4 times higher in African Americans compared to Caucasians (OR=4.0; 95% CI: 1.59-10.37). The odds of testing positive for at least one of the following STIs: chlamydia, gonorrhea, and trichomoniasis were 3.7 times higher for African-American teens. For every 1 year increase in age at conception, the odds of having tested positive for trichomoniasis decreased by 0.51 (OR=0.51; 95% CI: 0.26-0.95) and the odds of testing positive for gonorrhea decreased by 0.35 (OR=0.35; 95% CI: 0.16-0.80). Because maternal age and age at conception are highly correlated ($P<0.0001$), similar associations were found between maternal age and testing positive for trichomoniasis and gonorrhea (**Table 2.3**).

Table 2.3: Individual Risk Factors Associated with Maternal Infections and Inflammatory Conditions in 158 Pregnant Adolescents

Risk Factors	Odds Ratio (OR) (95% Confidence Interval)
Gonorrhea	
Maternal age (years)	0.38 (0.16-0.82)
Trichomoniasis	
Maternal age (years)	0.51 (0.26-0.92)
Chorioamnionitis	
Vitamin A <530 RAE ^a	3.71 (1.10-13.7)
Recto-vaginal Group B Strep	
Vitamin D intake (mcg/d)	0.87 (0.77-0.98)

^aRAE, retinoic acid equivalent. Logistic regression statistical analyses were performed.

Prior and current history of substance use was reported as follows: 32% (51/158) prior and 0.6% (1/158) current use of alcohol, 13% (21/158) prior and 11% (17/158) current use of cigarettes. In addition, 30% (48/158) and 3.2% (5/158) of teens reported use of marijuana or other drugs, respectively, sometime throughout their life. No significant associations were evident between drug and alcohol use and any of the outcomes monitored.

In this study population, approximately 50% of teens reported taking their standard prenatal supplements daily across gestation.(20) No significant relationship was observed between infection outcomes as a function of supplement use.

As previously published, the majority (70-90%) of these teens did not meet the EAR for several essential micronutrients.(20) No associations were found between macronutrient intake or water soluble nutrients and infection outcomes. However, low intake of two fat soluble vitamins (A and D) was associated with increased risk for placental chorioamnionitis and recto-vaginal GBS, respectively (**Table 2.3**).

Table 2.1 displays the distribution of ppBMI in the adolescents based on WHO classifications. Compared to underweight and normal weight teens, overweight and obese teens had a 3.3 times greater odds of testing positive for BV (OR=3.3; 95% CI: 1.28-9.31). Because of this association, additional risk factors for BV were also adjusted for ppBMI and findings remained statistically significant. These adjusted odds ratios are presented in **Table 2.4**.

Table 2.4: Maternal Risk Factors Associated with Selected Infections Across Gestation in 158 Pregnant Adolescents

Risk Factors	Adjusted Odds Ratio (AOR) (95% Confidence Interval)
Bacterial Vaginosis	
Maternal Race (African American)	4.61 (1.74-13.02)
Maternal ppBMI (kg/m ²) ^a	1.16 (1.04-1.30)

^appBMI, pre-pregnancy body mass index. Odds ratios were obtained using multiple logistic regression.

DISCUSSION

In this group of 158 pregnant adolescents, candida, bacterial vaginosis, recto-vaginal GBS, and cystitis were the four most commonly diagnosed infections. Because not all of these infections are routinely screened as part of standard obstetrical care and many are asymptomatic, our prevalence data likely underestimate the true prevalence of these infections in this pregnant adolescent population.

Limited data exist on STI prevalence in adolescent pregnant populations. Using the 2003-2004 NHANES data, we found that the estimated prevalence of STIs in our study population was higher than the weighted prevalence reported among sexually-experienced females in the U.S. aged 14-19 years for gonorrhea (3% vs. 2.5%), chlamydia (13% vs. 7.1%), and trichomoniasis (7.0% vs. 3.6%), but lower for genital herpes (1% vs. 3.4%) and human papillomavirus (HPV)

(7.6% vs. 29.5%).(22) The lower prevalence of HPV in these pregnant teens compared to the 2003-2004 national data is possibly a result of HPV vaccine administration in the U.S. since mid-2006 and changes in screening guidelines. In contrast, risk of BV in our study population was 25% higher than NHANES data (2001-2004) in which a 29.2% prevalence of BV was evident in women 14-49 years old.(12)

The prevalence of sexually transmitted infections such as chlamydia and gonorrhea is known to be higher among teens and young women.(23) Our findings have identified younger maternal age as a risk factor for infection with gonorrhea and trichomoniasis. These findings differ from national data that found older women (20-24 years) have a higher prevalence than adolescent females 15-19 years old,(24) and from NHANES 2003-2004 data that reported a significantly higher STI prevalence among 18-19 year old females when compared to 14-15 year old adolescent females.(22) Both of the national comparative data were not focused on pregnant adolescent populations. It is possible that disparities in STI prevalence exist between non-pregnant and pregnant adolescent populations and that the latter group may exhibit higher risk sexual behaviors especially at a younger age.

Finally 26% of teens in our study had a UTI (25% cystitis and 1% had pyelonephritis). Women, especially those who are pregnant are at an increased risk for UTIs.(25) Although asymptomatic bacteriuria (ASB) in non-pregnant women is relatively benign, ASB along with elevated progesterone levels during pregnancy can increase the risk of pyelonephritis.(26), (27) While ASB is thought to be present in 2-10% of all pregnant women,(28) prevalence data on acute cystitis is limited. Current estimates for symptomatic UTIs are limited but 1-3% of pregnant females are believed to have acute cystitis and 0.5-2% have acute pyelonephritis.(29)

No associations were found between the socioeconomic risk factors assessed and the

infection outcomes. Previous research has found that fewer years of education and having Medicaid insurance are associated with higher prevalence of BV among non-pregnant women.(30) We found no association between health insurance status and outcomes, which may be due to limited variability in the types of insurance used as 69% of teens with insurance used some form of Medicaid and only 10% reported no insurance. No data on family income was obtained which might have provided additional information on socioeconomic status.

In this study, African-American race was significantly correlated with increased risk of BV. A similar association between African-American race and BV was reported in a study of 298 non-pregnant women receiving care at a county health department site.(30) Using data from NHANES III, young adult African Americans have been shown to have a higher prevalence of chlamydia, gonorrhea, and trichomoniasis compared to whites.(31) We were unable to find an association between race and individual STIs but when these three STIs were combined into one category, the odds of testing positive for at least one of the three STIs was 3.7 times higher in African American teens.

Higher maternal ppBMI has been linked to increased risk of several adverse birth outcomes.(32) In this teen cohort, higher ppBMI was associated with increased risk BV. In non-pregnant women and men, obesity has been linked to a variety of infections: UTIs,(33) periodontitis,(34) and influenza A (H1N1).(35) Although the exact biological mechanisms underlying the observed associations between obesity and infection risk remain unclear, immune system dysregulation has been hypothesized.(36)

In these adolescents 38% had recto-vaginal colonization of GBS a higher prevalence than the 25% reported among U.S. pregnant women.(37) We observed an association between low maternal dietary vitamin D intake and increased odds of testing positive for recto-vaginal GBS

which may be due to the known effects of vitamin D on innate and adaptive immunity(38) and the lower vitamin D status found among minority pregnant women(39) and adolescents.(40)

Poor maternal intake of the vitamin A was associated with an increased risk of chorioamnionitis, an inflammation of the placenta due to bacterial infection. Vitamin A is a micronutrient widely believed to have anti-inflammatory effects,(41) and our findings with chorioamnionitis suggest that vitamin A may also be important in mediating pregnancy-specific inflammatory conditions.

Our retrospective, cross-sectional study had several limitations. We used self-reported dietary intake data which is subject to both under- and over-reporting and that may be more challenging to recall in an adolescent population. With this study design, we are unable to infer a causal relationship for any of the significant associations noted. Furthermore, it is difficult to describe the prevalence of these infections as being unique to pregnancy as some infections may have been contracted prior to pregnancy. Moreover, in teens diagnosed with multiple infections across gestation, it is not always known if the second diagnosis of the infection is a new incident infection or simply represents failure to effectively treat the prior infection as not all teens are re-tested post-treatment.

To our knowledge, this is the first study to explore risk factors for a wide range of diagnosed infections in a pregnant adolescent population receiving age-appropriate care from a prenatal adolescent clinic. This group of racially and ethnically diverse pregnant adolescents exhibited a higher prevalence of several sexually transmitted and vaginal infections compared to data from non-pregnant teen and adult populations. Several risk factors for infections and inflammatory conditions across pregnancy were identified including: African-American race, higher pre-pregnancy BMI, younger age at diagnosis, and low intake of fat-soluble vitamins A

and D. Further research is needed to understand the biological mechanisms which may be underlying the observed associations. These data may be useful for clinicians and sexual health educators who seek to reduce the infection burden among pregnant adolescents. In addition, these results support the need for promoting modifiable dietary and weight gain recommendations for improved maternal health.

REFERENCES

1. Sedgh G, Finer LB, Bankole A, Eilers MA, Singh S. Adolescent Pregnancy, Birth, and Abortion Rates Across Countries: Levels and Recent Trends. *J Adolesc Health* 2015;56(2):223-30.
2. Martin JA, Hamilton BE, Ventura SJ, Osterman MJ, Mathews TJ. Births: final data for 2011. *Natl Vital Stat Rep* 2013;62(1):1-69, 72.
3. Frost JJ, Singh S, Finer LB. Factors associated with contraceptive use and nonuse, United States, 2004. *Perspect Sex Reprod Health* 2007;39(2):90-9.
4. Shah PS, Balkhair T, Ohlsson A, Beyene J, Scott F, Frick C. Intention to become pregnant and low birth weight and preterm birth: a systematic review. *Matern Child Health J* 2011;15(2):205-16.
5. Chen XK, Wen SW, Fleming N, Demissie K, Rhoads GG, Walker M. Teenage pregnancy and adverse birth outcomes: a large population based retrospective cohort study. *Int J Epidemiol* 2007;36(2):368-73.
6. Weinstock H, Berman S, Cates W, Jr. Sexually transmitted diseases among American youth: incidence and prevalence estimates, 2000. *Perspect Sex Reprod Health* 2004;36(1):6-10.
7. Genc M, Ledger WJ. Syphilis in pregnancy. *Sex Transm Infect* 2000;76(2):73-9.
8. Cotch MF, Pastorek JG, 2nd, Nugent RP, Hillier SL, Gibbs RS, Martin DH, et al. *Trichomonas vaginalis* associated with low birth weight and preterm delivery. The Vaginal Infections and Prematurity Study Group. *Sex Transm Dis* 1997;24(6):353-60.
9. Mishori R, McClaskey EL, WinklerPrins VJ. Chlamydia trachomatis infections: screening, diagnosis, and management. *Am Fam Physician* 2012;86(12):1127-32.

10. Centers for Disease Control and Prevention. Morbidity and Mortality Weekly Report (MMWR). Sexually transmitted diseases treatment guidelines, 2010.
Available: <http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5912a1.htm>. Accessed December 20, 2014.
11. Hillier SL, Nugent RP, Eschenbach DA, Krohn MA, Gibbs RS, Martin DH, et al. Association between bacterial vaginosis and preterm delivery of a low-birth-weight infant. The Vaginal Infections and Prematurity Study Group. *N Engl J Med* 1995;333(26):1737-42.
12. Koumans EH, Sternberg M, Bruce C, McQuillan G, Kendrick J, Sutton M, et al. The prevalence of bacterial vaginosis in the United States, 2001-2004; associations with symptoms, sexual behaviors, and reproductive health. *Sex Transm Dis* 2007;34(11):864-9.
13. Smart S, Singal A, Mindel A. Social and sexual risk factors for bacterial vaginosis. *Sex Transm Infect* 2004;80(1):58-62.
14. ACOG educational bulletin. Antimicrobial therapy for obstetric patients. Number 245, March 1998 (replaces no. 117, June 1988). American College of Obstetricians and Gynecologists. *Int J Gynaecol Obstet* 1998;61(3):299-308.
15. Emiru T, Beyene G, Tsegaye W, Melaku S. Associated risk factors of urinary tract infection among pregnant women at Felege Hiwot Referral Hospital, Bahir Dar, North West Ethiopia. *BMC Res Notes* 2013;6:292.
16. Ikaheimo R, Siitonen A, Heiskanen T, Karkkainen U, Kuosmanen P, Lipponen P, et al. Recurrence of urinary tract infection in a primary care setting: analysis of a 1-year follow-up of 179 women. *Clin Infect Dis* 1996;22(1):91-9.
17. Scholes D, Hooton TM, Roberts PL, Stapleton AE, Gupta K, Stamm WE. Risk factors for recurrent urinary tract infection in young women. *J Infect Dis* 2000;182(4):1177-82.
18. Kontiokari T, Laitinen J, Jarvi L, Pokka T, Sundqvist K, Uhari M. Dietary factors protecting women from urinary tract infection. *Am J Clin Nutr* 2003;77(3):600-4.

19. Young BE, McNanley TJ, Cooper EM, McIntyre AW, Witter F, Harris ZL, et al. Vitamin D insufficiency is prevalent and vitamin D is inversely associated with parathyroid hormone and calcitriol in pregnant adolescents. *J Bone Miner Res* 2012;27(1):177-86.
20. Lee S, Young BE, Cooper E, Pressman E, Queenan RA, Olson C, Guillet R, O'Brien KO. Nutrient inadequacy is prevalent in pregnant adolescents and prenatal supplement use may not fully compensate for dietary deficiencies. *Infant, Child, & Adolescent Nutrition* 2014;6:152-159.
21. Physical status: the use and interpretation of anthropometry. Report of a WHO Expert Committee. *World Health Organ Tech Rep Ser* 1995;854:1-452.
22. Forhan SE, Gottlieb SL, Sternberg MR, Xu F, Datta SD, McQuillan GM, et al. Prevalence of sexually transmitted infections among female adolescents aged 14 to 19 in the United States. *Pediatrics* 2009;124(6):1505-12.
23. Satterwhite CL, Torrone E, Meites E, Dunne EF, Mahajan R, Ocfemia MC, et al. Sexually transmitted infections among US women and men: prevalence and incidence estimates, 2008. *Sex Transm Dis* 2013;40(3):187-93.
24. Centers for Disease Control and Prevention: STDs in adolescents and young adults 2013. Available: <http://www.cdc.gov/std/stats13/adol.htm>. Accessed January 11, 2015.
25. Foxman B. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. *Am J Med* 2002;113 Suppl 1A:5S-13S.
26. Schnarr J, Smaill F. Asymptomatic bacteriuria and symptomatic urinary tract infections in pregnancy. *Eur J Clin Invest* 2008;38 Suppl 2:50-7.
27. Jeyabalan A, Lain KY. Anatomic and functional changes of the upper urinary tract during pregnancy. *Urol Clin North Am* 2007;34(1):1-6.
28. Smaill F, Vazquez JC. Antibiotics for asymptomatic bacteriuria in pregnancy. *Cochrane Database Syst Rev* 2007(2):CD000490.

29. Matuszkiewicz-Rowinska J, Malyszko J, Wieliczko M. Urinary tract infections in pregnancy: old and new unresolved diagnostic and therapeutic problems. *Arch Med Sci* 2015;11(1):67-77.
30. Holzman C, Leventhal JM, Qiu H, Jones NM, Wang J. Factors linked to bacterial vaginosis in nonpregnant women. *Am J Public Health* 2001;91(10):1664-70.
31. Hallfors DD, Iritani BJ, Miller WC, Bauer DJ. Sexual and drug behavior patterns and HIV and STD racial disparities: the need for new directions. *Am J Public Health* 2007;97(1):125-32.
32. Abenhaim HA, Kinch RA, Morin L, Benjamin A, Usher R. Effect of prepregnancy body mass index categories on obstetrical and neonatal outcomes. *Arch Gynecol Obstet* 2007;275(1):39-43.
33. Semins MJ SA, Makary MA, Weiner J, Matlaga BR. The impact of obesity on urinary tract infection risk. *Urology* 2012;79(2):266-269.
34. Suvan J, D'Aiuto F, Moles DR, Petrie A, Donos N. Association between overweight/obesity and periodontitis in adults. A systematic review. *Obes Rev* 2011;12(5):e381-404.
35. Louie JK, Acosta M, Samuel MC, Schechter R, Vugia DJ, Harriman K, et al. A novel risk factor for a novel virus: obesity and 2009 pandemic influenza A (H1N1). *Clin Infect Dis* 2011;52(3):301-12.
36. Huttunen R, Syrjanen J. Obesity and the risk and outcome of infection. *Int J Obes (Lond)* 2013;37(3):333-40.
37. Centers of Disease Control and Prevention. Fast Facts. Group B Strep. Available: <http://www.cdc.gov/groupbstrep/about/fast-facts.html>. Accessed February 16, 2015.
38. Hewison M. An update on vitamin D and human immunity. *Clin Endocrinol (Oxf)* 2012;76(3):315-25.

39. Reeves IV, Bamji ZD, Rosario GB, Lewis KM, Young MA, Washington KN. Vitamin D deficiency in pregnant women of ethnic minority: a potential contributor to preeclampsia. *J Perinatol* 2014;34(10):767-73.
40. Davis LM, Chang SC, Mancini J, Nathanson MS, Witter FR, O'Brien KO. Vitamin D insufficiency is prevalent among pregnant African American adolescents. *J Pediatr Adolesc Gynecol* 2010;23(1):45-52.
41. Reifen R. Vitamin A as an anti-inflammatory agent. *Proc Nutr Soc* 2002;61(3):397-400.

CHAPTER 3

Vitamin D is Associated with Infections and Pro-Inflammatory Cytokines during Pregnancy

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ABSTRACT

Vitamin D is known to regulate innate and adaptive immune processes at the cellular level, but the role of vitamin D status on associated inflammatory processes across pregnancy is unclear. Our primary objective was to evaluate relationships between serum biomarkers of inflammation (interleukin (IL)-6, interleukin (IL)-10, tumor necrosis factor (TNF)- α , acute-phase proteins (C-reactive protein and hepcidin) and vitamin D status (25-hydroxyvitamin D (25(OH)D) and 1,25-dihydroxyvitamin D (1,25(OH) $_2$ D)) measured across pregnancy and in the neonate at birth. A second objective was to identify associations between vitamin D status and clinically diagnosed infections. In this cross-sectional study, 158 racially and ethnically diverse pregnant adolescents were recruited from the Rochester Adolescent Maternity Program (RAMP) in Rochester, NY. Serum 1,25(OH) $_2$ D was significantly lower in adolescents and neonates with IL-6 concentrations above the 75th percentile at delivery ($P=0.04$) and at birth ($P=0.004$), respectively. After adjusting for other potential covariates of inflammation, maternal serum 1,25(OH) $_2$ D was significantly positively associated with TNF- α during pregnancy ($P=0.02$), but at delivery 1,25(OH) $_2$ D and TNF- α were inversely associated with one another ($P=0.02$). Teens who tested positive for candida during pregnancy had significantly lower serum 25(OH)D ($P=0.04$) and 25(OH)D < 30 ng/ml was associated with bacterial vaginosis ($P=0.02$). African American exhibited significantly lower TNF- α concentrations at both mid-gestation ($P=0.05$) and delivery ($P<0.0001$) compared to the Caucasian adolescents. These results suggest that lower maternal vitamin D status may attenuate inflammatory responses and risk of infection across gestation.

INTRODUCTION

Cytokines are known to play an important role across gestation as they help regulate placental implantation, fetal allograft tolerance, and onset of labor.(1) Research conducted over the past 30 years suggests that cytokine production during pregnancy is altered in the presence of microbial infections.(2) The mother and/or fetus are thought to independently produce pro-inflammatory cytokines in response to systemic maternal infections and those which have reached the intra-amniotic cavity, respectively.(3) Abnormally high cytokine concentrations of interleukin (IL)-6 and tumor necrosis factor-alpha (TNF- α) measured in amniotic fluid, the amniochorionic membranes, or fetal plasma have been associated with adverse events during pregnancy, such as intra-amniotic microbial infection and spontaneous preterm labor in patients with premature rupture of membranes (PROM).(4, 5)

In addition to microbial infections, various demographic, anthropometric, and biological factors may influence cytokine concentrations across pregnancy. Older maternal age, higher body-mass-index (BMI), and prior pre-term delivery have been associated with higher plasma cytokine concentrations among pregnant European women (6) while a longer duration of labor has been shown to increase cord C-reactive protein (CRP) in newborns.(7) Active labor has been shown to increase maternal hepcidin concentrations,(8) and both cord IL-6 and hepcidin concentrations at birth.(9)

Calcitriol (1,25(OH)₂D) is believed to have a role in maintaining a balance between inflammation and immunosuppression.(10) In T helper cells (Th), *in vitro* studies in cultured peripheral blood mononuclear cells (PBMCs) from humans have found 1,25(OH)₂D acts to inhibit Th1 cell-associated pro-inflammatory cytokines (IL-2, interferon (IFN)- γ , etc.),(11) while other cell culture data in mice have found calcitriol to enhance the anti-inflammatory cytokines

produced by Th2 cells (IL-4, IL-5, etc.).(12) However, the *in vivo* action of 1,25(OH)₂D on cytokine production during unique human physiological states, such as pregnancy, is not well understood.

Due to the established anti-inflammatory activity of 1,25(OH)₂D *in vitro*, it is important to understand the vital role vitamin D may play in regulating inflammatory conditions and cytokine concentrations across gestation. The present research study was undertaken in a group of racially and ethnically diverse pregnant adolescents (≤ 18 years of age) previously shown to have both a high burden of infections across gestation (13) and low vitamin D status.(14) The primary objective of this research was to determine the relationship between serum biomarkers of vitamin D status ((25-hydroxyvitamin D (25(OH)D) and 1,25-dihydroxyvitamin D (1,25(OH)₂D)) and inflammation (IL-10, IL-6, TNF- α , CRP, and hepcidin) measured across pregnancy and in umbilical cord blood obtained from the neonate at birth. A second objective was to assess possible relationships between vitamin D status and clinically diagnosed infections during pregnancy.

MATERIALS AND METHODS

Study Population

Pregnant adolescents (n=158) between 13-18 years of age were enrolled in research studies designed to examine changes in maternal and fetal bone health, iron status and infections across pregnancy. Adolescents were recruited from the Rochester Adolescent Maternity Program (RAMP) in Rochester, New York. Pregnant adolescents were eligible to participate if between 12 and 30 weeks gestation at entry, carrying a single fetus, and otherwise healthy. At entry into the study, self-reported demographics, height, and weight were obtained by a study health project

coordinator. Pre-pregnancy body mass index (ppBMI) was calculated and categorized as underweight ($<18.5 \text{ kg/m}^2$), normal weight ($18.5\text{-}24.9 \text{ kg/m}^2$), overweight ($\geq 25.0 \text{ kg/m}^2$), or obese ($\geq 30.0 \text{ kg/m}^2$) using self-reported height and weight in accordance with the World Health Organization guidelines.(15) Mode of delivery, duration of labor, and neonatal birth data were abstracted from medical records. As previously reported, 18 clinically diagnosed infections were abstracted from the prenatal medical records by a study health project coordinator.(13) Informed written consent, and in those ≤ 14 y adolescent assent and parental consent were obtained. The study was approved by the Institutional Review Boards at Cornell University and the University of Rochester.

Biochemical Analyses

A non-fasting blood sample (10 mL) was collected at mid-gestation (average: 26 ± 3.4 weeks) (n=155) and upon admission to the hospital for delivery (average: 39.8 ± 1.3 weeks) (n=134). In addition, a 10 mL cord blood sample was obtained at delivery (n=128). Variability in sample size of biochemical markers analyzed in this study was due to missed sample collections or insufficient sample volume. Serum samples were stored at -80°C until analysis. The serum vitamin D status biomarker 25(OH)D was measured using a radioimmunoassay (RIA) (Diasorin Inc.), intact parathyroid hormone (iPTH) was measured by enzyme-linked immunoabsorbent assay (ELISA) (Diagnostic Systems Laboratories), and calcitriol ($1,25(\text{OH})_2\text{D}$) was measured by Dr. Michael Holick (Boston, MA) using an in-house thymus receptor binding assay. Data on biomarkers of vitamin D status and intact parathyroid hormone (iPTH) at mid-gestation, delivery, and in cord blood have been published.(14) Serum concentrations of iPTH $> 65 \text{ pg/mL}$ were defined as elevated. In accordance with the 2011 US Institute of Medicine's report on vitamin D and calcium,(16) we categorized 25(OH)D concentrations based on classifications

established to maintain adequate bone health: 25(OH)D \geq 20 ng/ml (“sufficient”) and $<$ 12 ng/ml (“at risk of deficiency”). Data were also analyzed using the Endocrine Society’s 25(OH)D cutoff for vitamin D sufficiency during pregnancy (25(OH)D \geq 30 ng/ml).(17)

Archived maternal serum samples were used to undertake analyses of cytokines (IL-6, IL-10, and TNF- α) in serum collected at mid-gestation, delivery, and in cord blood using the Magnetic Multiplex MAP kit (Millipore) in the Cornell Human Metabolic Research Unit (Ithaca, NY). The limit of detection (LOD) for IL-6, IL-10, and TNF- α was 0.13 pg/ml. The acute phase proteins, C-reactive protein (CRP) and hepcidin, were previously measured and reported.(18) High sensitivity CRP was analyzed using the Immulite 2000 immunoassay (Siemens), and hepcidin was measured by a competitive serum ELISA (Intrinsic Life Sciences). The LOD for CRP and hepcidin was 0.2 mg/L, and 2.5 ng/ml respectively. Additionally, the adipokine leptin was measured in serum with a commercially available ELISA from Millipore as reported.(14)

Statistical Analyses

Concentrations of biomarkers are presented either as the mean \pm SD or geometric mean (95% confidence interval (CI)), percentage of values above/below the established cut-offs or LOD (if applicable), or percentage of elevated values ($>$ 75th percentile) relative to the sample population. Undetectable values were included in all analyses and computed as $\frac{1}{2}$ x LOD. Differences in mean concentrations of serum biomarkers across gestation were calculated using ANOVA with Tukey honest significant difference (HSD) test for multiple comparisons. Simple linear regression was used to identify correlations between inflammatory biomarkers. Possible determinants of maternal inflammation were explored (maternal vitamin D status, race, ethnicity, gestational age, ppBMI, serum leptin, and the presence of a clinically diagnosed infection during pregnancy) using linear regression or t-tests. Likewise, potential determinants of neonatal

inflammation (neonatal vitamin D status, mode of delivery, duration of labor, serum leptin, maternal gestational age, a clinically diagnosed maternal infection during pregnancy, and maternal inflammatory biomarker concentrations) were assessed. To determine the most significant predictors of inflammation, backwards stepwise regression models were constructed using predictors associated with inflammation in bivariate analyses ($P<0.10$). Natural log (Ln) transformations were applied to non-normally distributed variables. Results were considered statistically significant at $P<0.05$. All analyses were performed using JMP 12.0 (SAS Institute).

TABLE 3.1: Subject Characteristics of the 158 Pregnant Adolescents¹

Characteristics	Value	Range
Age at enrollment, years (n)	17.1 ± 1.1 (158)	13.6 – 18.7
Maternal Race, % (n)		
African American	63.3 (158)	
Caucasian	35.4 (158)	
Native American	1.3 (158)	
Ethnicity, % (n)		
Hispanic	24.7 (158)	
Non-Hispanic	75.3 (158)	
GA at prenatal care entry, weeks (n)	10.5 ± 4.7 (137)	2 – 24
GA at delivery, weeks (n)	39.2 ± 3.0 (154)	21 – 42.1
Pre-pregnancy BMI, kg/m ² (n)	24.7 ± 5.4 (156)	15.0 – 42.1
Mode of Delivery		
C-Section, % (n)	12 (150)	
Vaginal, % (n)	88 (150)	
Duration of Labor, hours	8.50 ± 5.4 (133)	0.02 – 24.1
Preterm Birth < 37 weeks, % (n)	9.1 (154)	
Low birthweight < 2500 g, % (n)	3208 ± 2398 (150)	1054 – 4705
Diagnosed Maternal Infections, % (n)		
Bacteria Vaginosis	40% (158)	
Recto-vaginal GBS	38% (158)	
Candida	42% (158)	
Chlamydia	13% (158)	

GA, gestational age; BMI, body-mass-index; GBS, group B streptococcus. ¹Values are presented as the mean ± SD (n) or % (n).

RESULTS

Subject Characteristics

Subject characteristics are presented in **Table 3.1**. Over 60% of the teens self-identified as African American and over 30% self-identified as Caucasian which is similar to the U.S. teen pregnancy rate which is twice as high for non-Hispanic blacks compared to non-Hispanic white teens 15-19 years old (43.9 vs 20.5 per 1,000 females).(19) While receiving age-specific care at RAMP, our study cohort had a lower rate of Cesarean (C-section) deliveries compared to U.S. data in women < 20 y (12% vs. 22%), and lower rates of preterm birth (< 37 weeks) (9% vs. 13%), and a lower rate of babies born low birth weight (LBW < 2500 g) (6.7% vs 9.3%) compared to U.S. teens 15- 19 years old.(19) These data are consistent with the prenatal care provided at midwife-run clinics.

Relevant clinically diagnosed infections in this cohort are listed in **Table 3.1**. Chorioamnionitis was documented in 14% of the placentas that were sent to pathology (n=45). As previously described, the infection burden of this population was more than 25% higher compared to non-pregnant and pregnant female adolescents and also compared to adult women for chlamydia, bacterial vaginosis (BV), and recto-vaginal group B streptococcus (GBS).(13) Racial differences were evident such that African American pregnant adolescents in this cohort were more likely to test positive for bacterial vaginosis (BV) or at least one of the following STIs: chlamydia, gonorrhea, and trichomoniasis when compared to Caucasian adolescents.(13)

Biomarkers of Vitamin D and Inflammation

Similar to data collected in pregnant adolescents (13-18 years old),(14, 20) prevalence of 25(OH)D < 20 ng/ml was high among the adolescents and neonates studied (47-50%) (**Table 3.2**). No significant difference in mean mid-gestation 25(OH)D was noted between ppBMI

categories. Additionally, the mean 1,25(OH)₂D concentrations remained high (> 100 pg/ml) across pregnancy.(14)

TABLE 3.2: Markers of Vitamin D Status and Inflammation in Pregnant Adolescents and Their Neonates at Birth

	N	Mid-gestation	N	Delivery	N	Cord
25(OH)D, ng/ml¹	155	20.2 (18.8, 21.8)	133	18.6 (17.0, 20.5)	121	18.8 (17.1, 20.5)
<12 ng/ml, % (n)		10		17		17
<20 ng/ml, % (n)		50		48		47
<30 ng/ml, % (n)		81		81		81
1,25(OH)₂D, pg/ml¹	100	117 ± 30.3 ^a	96	106 ± 30.6 ^b	74	44.7 (41.3, 48.4) ^c
iPTH , pg/ml¹	91	25.0 (22.3, 27.9) ^a	80	38.4 (33.8, 43.6) ^b	33	19.0 ± 12.1 ^c
> 65 pg/ml, % (n)		3.3		18		0
IL-10, pg/ml¹	144	4.57 (3.18, 6.57) ^a	125	9.65 (7.22, 12.9) ^b	96	18.0 (13.6, 24.0) ^c
≤ 0.13 pg/ml, %		17.4		5.6		3.1
TNF-α, pg/ml¹	145	4.48 (3.92, 5.14) ^a	126	3.54 (3.17, 3.97) ^b	97	8.31 (7.40, 9.33) ^c
≤ 0.13 pg/ml, %		0.7		0		0
IL-6, pg/ml¹	145	1.02 (0.78, 1.33) ^a	126	3.73 (3.01, 4.63) ^b	97	8.41 (5.99, 11.7) ^c
≤ 0.13 pg/ml, %		15		2.4		0
CRP, mg/L²	113	3.78 (3.10, 4.66) ^a	4	2.19 (0.06, 79.4)	86	0.17 (0.13, 0.23) ^b
≤ 2.0 mg/L, %		0.9		25		79
Hepcidin, ng/ml¹	145	20.7 (18.0, 23.7) ^a	129	24.2 (20.2, 29.1) ^a	120	94.2 (80.4, 110) ^b
≤ 2.5 ng/ml, %		4.8		7.8		0
Leptin, µg/L¹	141	22.3 (19.7, 25.2) ^a	127	29.4 (25.7, 33.6) ^b	114	7.47 (6.26, 8.91) ^c

25(OH)D, 25-hydroxyvitamin D; 1,25(OH)₂D, 1,25-dihydroxyvitamin D; iPTH, intact parathyroid hormone, IL, interleukin; TNF, tumor necrosis factor; CRP, C-reactive protein.

¹Values are presented as geometric mean (95% CI) except for 1,25(OH)₂D (mid-gestation and delivery) and iPTH (cord) which are presented as mean ± SD. ²Only 4 subjects had CRP values at delivery and these data were excluded from all analyses due to insufficient sample size. Letter superscripts indicate statistically significant differences between mean mid-gestation, delivery, and cord values (*P*<0.05).

Serum cytokine concentrations at mid-gestation and delivery are displayed in **Table 3.2**. At mid-gestation, IL-6 was significantly positively associated with CRP ($r=0.28$, $n=111$, $P=0.003$) (data not shown). At mid-gestation, positive trends were observed between hepcidin and the cytokines IL-6 ($r=0.16$, $n=143$, $P=0.05$) and TNF- α ($r=0.15$, $n=143$, $P=0.08$). Elevated CRP (> 5 mg/L) at mid-gestation was evident in 42% of the teens, and the mid-gestation geometric mean leptin concentration was significantly higher in those with elevated CRP concentrations compared to those without (32.7 $\mu\text{g/L}$, $n=43$ vs. 17.5 $\mu\text{g/L}$, $n=64$, $P<0.0001$). The geometric mean mid-gestation leptin concentration was significantly lower in those with mid-gestation IL-10 above the 75th percentile compared to those below (17.9 $\mu\text{g/L}$, $n=34$ vs. 24.1 $\mu\text{g/L}$, $n=104$, $P=0.04$). Maternal mid-gestation leptin was significantly positively correlated with IL-6 ($r=0.18$, $n=139$, $P=0.03$). At delivery, positive trends between hepcidin and both IL-6 ($r=0.16$, $n=125$, $P=0.07$) and IL-10 ($r=0.17$, $n=124$, $P=0.06$) were evident with the former previously shown to achieve statistical significance in the larger cohort.(18) In addition, a positive trend between IL-10 and hepcidin was evident ($r=0.17$, $n=124$, $P=0.06$) at delivery.

Relationships between maternal and neonatal cytokines were explored. In the neonate, geometric mean cytokine concentrations were significantly higher than maternal concentrations for IL-6, IL-10, TNF- α and hepcidin, but significantly lower for CRP (**Table 3.2**). Similar to patterns observed in the mother, positive linear correlations between inflammatory biomarkers were also evident within the neonate (**Table 3.3**). In cord blood, IL-6 was significantly positively associated with CRP ($r=0.29$, $n=71$, $P=0.01$) (data not shown). No significant associations were found between cord leptin and the inflammatory biomarkers in cord blood. Maternal IL-6 during pregnancy was significantly inversely associated with umbilical cord IL-10 ($r= -0.23$, $n=88$, $P=0.03$). A positive relationship between maternal mid-gestation TNF- α and umbilical cord

TNF- α approached significance ($r=0.21$, $n=89$, $P=0.05$). Significant positive correlations were evident between maternal IL-6 at delivery and umbilical cord CRP ($r=0.26$, $n=82$, $P=0.02$).

Consistent with findings evaluating determinants of iron status in the larger cohort,(9) maternal mid-gestation hepcidin was significantly positively correlated with cord hepcidin ($r=0.30$, $n=110$, $P=0.001$).

TABLE 3.3: Correlations between Inflammation Biomarkers in Maternal Serum Collected during Pregnancy and at Delivery and in Cord Serum Obtained at Birth¹

	IL-6	TNF- α	Hepcidin
Pregnancy (~26 weeks)			
IL-10	$r=0.47^{***}$ $n=144$	$r=0.30^{***}$ $n=144$	
IL-6		$r=0.44^{***}$ $n=145$	
Delivery			
IL-10	$r=0.49^{***}$ $n=125$	$r=0.20^*$ $n=125$	
IL-6		$r=0.32^{***}$ $n=126$	
Neonatal Cord			
IL-10	$r=0.44^{***}$ $n=96$	$r=0.38^{***}$ $n=96$	$r=0.22^*$ $n=95$
IL-6		$r=0.29^{**}$ $n=97$	$r=0.38^{***}$ $n=96$
TNF- α			$r=0.37^{***}$ $n=96$

IL, interleukin; TNF, tumor necrosis factor. ¹All biomarkers were natural log (Ln) transformed. Data presented as Correlation Coefficients (r) and sample size (n). * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

We explored possible relationships between maternal labor or delivery variables and neonatal inflammatory biomarkers. Greater mean duration of labor was associated with cord hepcidin above the 75th percentile ($P=0.01$), but not with CRP or any other pro-inflammatory cytokine measured. Of the cord inflammatory biomarkers measured, only cord TNF- α was

significantly impacted by mode of delivery as evidenced by the significantly higher geometric mean concentration of cord TNF- α in neonates born via vaginal delivery compared to C-section ($P=0.0008$).

Demographic and Anthropometric Risk Factors

Of the demographic variables explored (race, ethnicity and gestational age), race was the only significant predictor of inflammation. Caucasian teens ($n=46$) exhibited a 2.75-times higher risk of having elevated TNF- α at delivery when compared to African-American teens ($n=80$) [relative risk, RR: 2.75, (95% CI: 1.47, 5.15)]. There was a positive trend between gestational age at delivery and the concentration of hepcidin at delivery ($P=0.06$). At mid-gestation, the difference in TNF- α between African American and Caucasian teens approached significance ($P=0.05$). Compared to teens who were underweight or normal weight, teens that were overweight or obese prior to pregnancy were at a significantly higher risk for having mid-gestation IL-6 [RR: 1.75 (95% CI: 1.002, 3.06)] and TNF- α [RR: 1.85 (95% CI: 1.05, 3.27)] above the 75th percentile as well as CRP ≥ 5 mg/L [RR: 2.83 (95% CI: 1.79, 4.47)].

Associations between Infections and Vitamin D

Teens with 25(OH)D < 30 ng/ml at mid-gestation ($n=72$) were significantly more likely to have a positive diagnosis of bacterial vaginosis (BV) ($P=0.02$, $n=53$). In addition, those who tested positive for BV at any point during gestation had significantly higher mean concentrations of serum calcitriol at delivery ($P=0.007$, $n=40$) while a positive trend between calcitriol and infection with BV was evident at mid-gestation ($P=0.08$, $n=41$). Because maternal race was previously shown to be associated with BV in this cohort,(13) we noted that while controlling for maternal race, the associations between positive BV infection and vitamin D status (mid-gestation 25(OH)D < 30 ng/ml ($P=0.06$)) and delivery 1,25(OH) $_2$ D ($P=0.03$)) were found to be

less significant. Pregnant teens who tested positive for candida compared to those who tested negative for candida had significantly lower geometric mean serum 25(OH)D concentrations measured at mid-gestation (19.5 ng/ml, n=67 vs. 23.8 ng/ml, n=30, $P=0.04$) and at delivery (17.2 ng/ml, n=56 vs. 23.8 ng/ml, n=26, $P=0.02$).

Relationships between Infections and Inflammation

Diagnosis of bacterial vaginosis at any point during pregnancy was associated with a significantly higher mid-gestation geometric mean hepcidin concentration (22.1 ng/ml, n=57 vs. 13.8 ng/ml, n=29, $P=0.01$). Higher geometric mean maternal IL-6 concentrations at delivery were weakly associated with either a diagnosis of chorioamnionitis (4.9 pg/ml, n= 17 vs. 2.2 pg/ml, n=15, $P=0.04$) or chlamydia (6.2 pg/ml, n=19 vs. 3.4 pg/ml, n=107, $P=0.049$). Likewise, having a delivery geometric mean hepcidin concentration above the 75th percentile was significantly associated with a diagnosis of chorioamnionitis (n=8, $P=0.004$).

Significant associations between maternal infections across pregnancy and neonatal inflammation at birth were also noted. Neonates whose mother tested positive for chlamydia had a 2.8-times higher risk of having cord TNF- α above the 75th percentile [RR: 2.82 (95% CI: 1.49, 5.35)]. A significantly higher geometric mean cord hepcidin concentration was observed in neonates whose mother tested positive for chlamydia at any point during pregnancy (141 ng/ml, n=17 vs. 88.1, n=103, $P=0.04$). The geometric mean IL-10 concentration in neonates whose mother tested positive for recto-vaginal group B streptococcus (GBS) was significantly lower compared to those testing negative for this infection (12.2 pg/ml, n=41, vs. 24.2 pg/ml, n=53, $P=0.02$).

Associations between Inflammation and Vitamin D

Maternal mid-gestation 25(OH)D was significantly positively associated with delivery TNF- α ($P=0.02$). Teens with mid-gestation 25(OH)D < 12 ng/ml had a significantly higher geometric mean hepcidin concentration (31.1 ng/ml, $n=16$, vs. 19.6 ng/ml, $n=129$, $P=0.04$). There was a significant positive association between mid-gestation 1,25(OH) $_2$ D and mid-gestation TNF- α ($P=0.005$, $n=100$). At mid-gestation, the mean 1,25(OH) $_2$ D concentration was higher in teens with mid-gestation IL-6 concentrations above the 75th percentile (134 pg/ml, $n=25$ vs. 111 pg/ml, $n=75$, $P=0.001$) and mid-gestation CRP concentrations ≥ 5 mg/L (127 pg/ml, $n=34$ vs. 111 pg/ml, $n=52$, $P=0.01$), respectively. In contrast, at delivery the mean 1,25(OH) $_2$ D concentration at delivery was significantly lower in teens with delivery IL-6 (96.6 pg/ml, $n=26$ vs. 111 pg/ml, $n=69$, $P=0.04$) and delivery TNF- α (85.6 pg/ml, $n=19$ vs. 112 pg/ml, $n=76$, $P=0.0005$) concentrations above the 75th percentile, respectively. Using multivariate modeling, a significant interaction effect ($P=0.02$, $n=55$) was observed between BV and 1,25(OH) $_2$ D such that the inverse association between 1,25(OH) $_2$ D and log IL-6 was only significant in those that tested positive for BV.

In neonates, there was a positive correlation between cord 25(OH)D and TNF- α ($P=0.04$, $n=94$). Neonates with umbilical cord 25(OH)D < 12 ng/ml had significantly lower geometric mean cord hepcidin concentration (58.8 ng/ml, $n=19$ vs. 105 ng/ml, $n=97$, $P=0.006$). The 1,25(OH) $_2$ D umbilical cord geometric mean concentration was significantly lower in those with cord IL-6 (37.1 pg/ml, $n=16$ vs. 49.0 pg/ml, $n=50$, $P=0.004$) and hepcidin (38.0 ng/ml, $n=20$, vs. 47.5 ng/ml, $n=54$, $P=0.01$) above the 75th percentile, respectively.

Finally, **Table 3.4** displays multivariate models used to assess whether vitamin D remained a significant predictor of maternal inflammatory biomarkers at mid-gestation, delivery,

and in the neonate at birth while controlling for covariates determined to be significant from prior bivariate analyses.

TABLE 3.4: Multivariate Models for Selected Inflammation Markers¹

Variables	<i>P</i> value	R ² Adj.	β
Ln Mid-gestation IL-6		0.33	
Ln Mid-gestation Leptin	0.005		0.428
Ln Mid-gestation IL-10	<0.0001		0.259
Ln Mid-gestation TNF-α	<0.0001		0.660
Ln Mid-gestation TNF-α		0.23	
Mid-gestation 1,25(OH) ₂ D	0.02		0.005
Ln Mid-gestation IL-6	0.03		0.109
Ln Mid-gestation IL-10	0.06		0.068
African American (ref: CA)	0.009		-0.365
Ln Mid-gestation CRP		0.37	
Mid-gestation 1,25(OH) ₂ D	0.09		0.005
Ln Mid-gestation Leptin	0.003		0.454
Ln ppBMI (kg/m ²)	0.001		1.65
Ln Delivery TNF-α		0.24	
Delivery 1,25(OH) ₂ D	0.02		-0.005
Ln Delivery IL-6	0.03		0.113
African American (ref: CA)	0.001		-0.405
Ln Cord Hepcidin		0.47	
Ln Cord 25(OH)D	0.09		0.320
Ln Cord 1,25(OH) ₂ D	<0.0001		-1.16
Ln Mid-gestation Hepcidin	0.002		0.327
Ln Cord IL-6	0.02		0.123

Ln, natural log; IL, interleukin; TNF, tumor necrosis factor; 1,25(OH)₂D, 1,25-dihydroxyvitamin D; ref, reference level; CA, Caucasian; CRP, C-reactive protein; ppBMI, pre-pregnancy body-mass-index; 25(OH)D, 25-hydroxyvitamin D. ¹Bold-face indicates statistical significance (*P*<0.05).

DISCUSSION

In these pregnant adolescents, teens with lower 25(OH)D concentrations were at greater risk of infections (candida and bacterial vaginosis) during pregnancy. Adolescents with chlamydial infections exhibited increased inflammation at delivery, a finding that was associated with decreased calcitriol (1,25(OH)₂D). Temporal changes in the associations between vitamin D and inflammatory outcomes from mid-gestation to delivery may be a consequence of the

dynamic changes in inflammation and calcitriol that occur across gestation. Maternal infection with chlamydia was associated with elevated hepcidin and TNF- α in cord blood. Because elevated inflammation has been associated with increased risk of adverse birth outcomes, and maternal nutritional status may attenuate inflammatory responses across gestation, more normative data on maternal vitamin D status and inflammation and its impact on the neonate at birth are needed.

The mean mid-gestation IL-6 concentration observed in this cohort at ~26 weeks of gestation was similar to mid-gestation (22-24 weeks) values reported in a Texas study of 28 pregnant teens (< 18 y) and 438 pregnant adult women (~1.0 pg/ml)(21) whereas mean mid-gestation TNF- α concentrations in our study population were significantly higher than those previously noted in adult pregnant women from Texas (~2 pg/ml).(21) The significant positive correlations we observed between IL-6 and TNF- α at mid-gestation are consistent with the positive correlations noted at 24 weeks of gestation in a study of over 100,000 pregnant European women using a multiplex flow cytometric assay.(6)

African American teens in our study exhibited significantly lower TNF- α at both mid-gestation and delivery when compared to the Caucasian adolescents. This finding is supported by other reports in the literature that have reported genetic differences in cytokine genes and receptors between Caucasian and African Americans. For example, unique single nucleotide polymorphisms in TNF- α and its receptor have been reported among Caucasians and African Americans for cytokines such as TNF- α and their corresponding receptors.(22, 23) The significantly lower soluble TNF- α concentrations in the African American adolescents that we observed is also consistent with a previous study of fetal membranes which showed that the concentrations of soluble TNF receptors (measured using a multiplex immunoassay) were

significantly decreased in culture media from African Americans but increased in culture media from Caucasians following *in vitro* lipopolysaccharide (LPS) stimulation.(24)

We observed significantly higher mid-gestation IL-6, TNF- α , and CRP concentrations in teen mothers with higher ppBMIs. These findings are consistent with the positive correlation observed between ppBMI and IL-6 and CRP that we previously reported in the larger cohort.(25) Similarly, other studies have demonstrated higher IL-6 in obese individuals,(26) and pregnant obese mice,(27) compared to lean control groups. Elevated TNF- α in non-pregnant adults has been hypothesized to play a role in obesity-induced insulin resistance.(28, 29)

In these pregnant adolescents, vitamin D status was independently associated with clinical diagnosis of both a bacterial (BV) and a fungal infection (candida). Similar to other research findings conducted in pregnant women, we found an inverse association between serum 25(OH)D at mid-gestation and BV.(30) In contrast, we observed higher serum 1,25(OH)₂D at mid-gestation and at delivery in those with BV infection. These findings are consistent with previously published findings in the larger cohort which noted an inverse association between mid-gestation 25(OH)D and delivery 1,25(OH)₂D.(14) The inverse relationship between IL-6 and 1,25(OH)₂D evident at delivery appeared to be moderated by the presence of BV infection suggesting that the anti-inflammatory role of 1,25(OH)₂D during pregnancy may be more pronounced in those with infections. In addition, we found that lower maternal 25(OH)D status during pregnancy and at delivery was associated with a positive diagnosis of candida infection which is consistent with results from a study of hospitalized adults.(31) Our results suggest that the immuno-modulatory role of vitamin D may be more evident during states of infection and that lower vitamin D status may reduce one's ability to clear infections during pregnancy.

Calcitriol was significantly positively associated with TNF- α during pregnancy but it was inversely associated with TNF- α in delivery sera. Prior studies have found inverse associations between 1,25(OH) $_2$ D and TNF- α in cultured trophoblasts obtained from placentas collected during uncomplicated and pre-eclamptic pregnancies.(32, 33) The positive association we observed may be due to differential regulation of cytokines at the systemic versus the cellular level or possibly due to differences between *in vivo* conditions and *in vitro* model systems.

Temporal changes in 1,25(OH) $_2$ D but not 25(OH)D across pregnancy are important to take into account when evaluating interactions between vitamin D and cytokines. While 25(OH)D geometric mean concentrations did not significantly change between pregnancy and delivery, mean 1,25(OH) $_2$ D decreased by ~ 9% from mid-gestation to delivery. Maternal 1,25(OH) $_2$ D is also dependent on 25(OH)D status, and a vitamin D supplementation study conducted in 350 pregnant women found linear relationships between 25(OH)D and 1,25(OH) $_2$ D up until 25(OH)D concentrations of 40 ng/mL were achieved,(34) a concentration that most teens did not achieve in our study. A recent RCT conducted in 57 US pregnant women found that 2000 IU of vitamin D supplementation was more effective in increasing IL-10 $^{+}$ regulatory CD4 $^{+}$ T cells later in pregnancy (36 weeks gestation) than 400 IU of vitamin D.(35) Taken together with our findings which demonstrated an inverse association between maternal calcitriol and pro-inflammatory cytokines IL-6 and TNF- α at delivery, these results suggest that the anti-inflammatory action of vitamin D may be more critical later in pregnancy.

Both the pregnant adolescent and her fetus are capable of independently synthesizing calcitriol and mounting inflammatory responses, but little is known about how maternal and fetal cytokines may interact. Although we did not observe significant correlations between maternal and fetal cytokines of the same class, data on the ability of cytokines to cross the placenta are

conflicting. A previous study of 19 normal-term placentas found that cytokines TNF- α , IL-1, and IL-6 did not cross the placenta ex vivo suggesting that the inflammatory response detected in cord blood is of fetal origin.(36) In contrast, an ex vivo study of 10 normal-term placentas found that IL-6 was transported across the placenta in a bi-directional manner.(37) The stage of gestation may impact findings as data obtained in a rat model found that maternal IL-6 readily crossed the rat placenta at mid-gestation but transfer was reduced by late gestation.(38)

Chlamydia is a bacterial infection that the neonate is capable of contracting while passing through the birth canal at delivery.(39) In neonates studied, maternal infection with chlamydia was associated with a significantly increased concentration of TNF- α and hepcidin. A study conducted in newborn mice demonstrated that the passive transfer of TNF- α from an immunized adult mice to the fetus in utero was associated with a significant decrease in postnatal chlamydial colonization of the newborn murine lung.(40)

Several limitations in our study design were evident. Since no normative data on mean serum cytokine concentrations are available and there are no adjustment factors for normalizing data to account for variable analytical approaches, we could not directly compare our mean cytokine values to those reported in other similar populations. Although we did detect several significant associations between maternal biomarkers (inflammation and vitamin D status) and infection status, our retrospective study design did not allow us to establish causality.

CONCLUSIONS

Despite conflicting evidence describing the impact of vitamin D status on inflammation during pregnancy, we observed significant inverse associations between 1,25(OH) $_2$ D and IL-6 and TNF- α in the mother at delivery and between 1,25(OH) $_2$ D and IL-6 and hepcidin in the

neonate at birth. We also demonstrated that the presence of bacterial vaginosis impacted the association between IL-6 and 1,25(OH)₂D at delivery. Together, our results suggest that 1,25(OH)₂D may influence changes in pro-inflammatory cytokine production during pregnancy and infections may moderate these relationships. Given that elevated inflammatory biomarkers have been linked to adverse pregnancy outcomes, more research is needed to determine if vitamin D supplementation can modify maternal inflammatory responses and promote healthy birth outcomes in pregnant populations, particularly those at increased risk of infections and vitamin D insufficiency.

REFERENCES

1. Saito S. Cytokine cross-talk between mother and the embryo/placenta. *J Reprod Immunol* 2001;52(1-2):15-33.
2. Romero R, Erez O, Espinoza J. Intrauterine infection, preterm labor, and cytokines. *J Soc Gynecol Investig* 2005;12(7):463-5.
3. Romero R, Brody DT, Oyarzun E, Mazor M, Wu YK, Hobbins JC, et al. Infection and labor. III. Interleukin-1: a signal for the onset of parturition. *Am J Obstet Gynecol* 1989;160(5 Pt 1):1117-23.
4. Santhanam U, Avila C, Romero R, Viguet H, Ida N, Sakurai S, et al. Cytokines in normal and abnormal parturition: elevated amniotic fluid interleukin-6 levels in women with premature rupture of membranes associated with intrauterine infection. *Cytokine* 1991;3(2):155-63.
5. Romero R, Gomez R, Ghezzi F, Yoon BH, Mazor M, Edwin SS, et al. A fetal systemic inflammatory response is followed by the spontaneous onset of preterm parturition. *Am J Obstet Gynecol* 1998;179(1):186-93.
6. Curry AE, Vogel I, Skogstrand K, Drews C, Schendel DE, Flanders WD, et al. Maternal plasma cytokines in early- and mid-gestation of normal human pregnancy and their association with maternal factors. *J Reprod Immunol* 2008;77(2):152-60.
7. Logan CA, Thiel L, Bornemann R, Koenig W, Reister F, Brenner H, et al. Delivery Mode, Duration of Labor, and Cord Blood Adiponectin, Leptin, and C-Reactive Protein: Results of the Population-Based Ulm Birth Cohort Studies. *PLoS One* 2016;11(2):e0149918.
8. Rehu M, Punnonen K, Ostland V, Heinonen S, Westerman M, Pulkki K, et al. Maternal serum hepcidin is low at term and independent of cord blood iron status. *Eur J Haematol* 2010;85(4):345-52.

9. Lee S, Guillet R, Cooper EM, Westerman M, Orlando M, Kent T, et al. Prevalence of anemia and associations between neonatal iron status, hepcidin, and maternal iron status among neonates born to pregnant adolescents. *Pediatr Res* 2016;79(1-1):42-8.
10. Hewison M. Vitamin D and immune function: an overview. *Proc Nutr Soc* 2012;71(1):50-61.
11. Rigby WF, Denome S, Fanger MW. Regulation of lymphokine production and human T lymphocyte activation by 1,25-dihydroxyvitamin D3. Specific inhibition at the level of messenger RNA. *J Clin Invest* 1987;79(6):1659-64.
12. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A. 1 α ,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. *J Immunol* 2001;167(9):4974-80.
13. Akoh CC, Pressman EK, Cooper E, Queenan RA, Pillittere J, O'Brien KO. Prevalence and Risk Factors for Infections in a Pregnant Adolescent Population. *J Pediatr Adolesc Gynecol* 2016 (*In Press*)
14. Young BE, McNanley TJ, Cooper EM, McIntyre AW, Witter F, Harris ZL, et al. Vitamin D insufficiency is prevalent and vitamin D is inversely associated with parathyroid hormone and calcitriol in pregnant adolescents. *J Bone Miner Res* 2012;27(1):177-86.
15. World Health Organization. Physical status: the use and interpretation of anthropometry. Report of a WHO Expert Committee. World Health Organization *Technical Report Series* 1995;854:1-452.
16. Institute of Medicine. Dietary reference intakes for calcium and vitamin d. *National Academy of Sciences Press*. Washington (DC), 2011.
17. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab* 2011;96(7):1911-30.

18. Lee S, Guillet R, Cooper EM, Westerman M, Orlando M, Pressman E, et al. Maternal inflammation at delivery affects assessment of maternal iron status. *J Nutr* 2014;144(10):1524-32.
19. Martin JA, Hamilton BE, Osterman MJ, Curtin SC, Matthews TJ. Births: final data for 2012. *Natl Vital Stat Rep* 2013;62(9):1-68.
20. Davis LM, Chang SC, Mancini J, Nathanson MS, Witter FR, O'Brien KO. Vitamin D insufficiency is prevalent among pregnant African American adolescents. *J Pediatr Adolesc Gynecol* 2010;23(1):45-52.
21. Ruiz RJ, Jallo N, Murphey C, Marti CN, Godbold E, Pickler RH. Second trimester maternal plasma levels of cytokines IL-1Ra, IL-6 and IL-10 and preterm birth. *J Perinatol* 2012;32(7):483-90.
22. Van Dyke AL, Cote ML, Wenzlaff AS, Land S, Schwartz AG. Cytokine SNPs: Comparison of allele frequencies by race and implications for future studies. *Cytokine* 2009;46(2):236-44.
23. Menon R, Velez DR, Morgan N, Lombardi SJ, Fortunato SJ, Williams SM. Genetic regulation of amniotic fluid TNF-alpha and soluble TNF receptor concentrations affected by race and preterm birth. *Hum Genet* 2008;124(3):243-53.
24. Menon R, Thorsen P, Vogel I, Jacobsson B, Williams SM, Fortunato SJ. Increased bioavailability of TNF-alpha in African Americans during in vitro infection: predisposing evidence for immune imbalance. *Placenta* 2007;28(8-9):946-50.
25. Cao C, Pressman EK, Cooper EM, Guillet R, Westerman M, O'Brien KO. Placental heme receptor LRP1 correlates with the heme exporter FLVCR1 and neonatal iron status. *Reproduction* 2014;148(3):295-302.
26. Roytblat L, Rachinsky M, Fisher A, Greemberg L, Shapira Y, Douvdevani A, et al. Raised interleukin-6 levels in obese patients. *Obes Res* 2000;8(9):673-5.

27. Sanders TR, Kim DW, Glendining KA, Jasoni CL. Maternal obesity and IL-6 lead to aberrant developmental gene expression and deregulated neurite growth in the fetal arcuate nucleus. *Endocrinology* 2014;155(7):2566-77.
28. Nieto-Vazquez I, Fernandez-Veledo S, Kramer DK, Vila-Bedmar R, Garcia-Guerra L, Lorenzo M. Insulin resistance associated to obesity: the link TNF-alpha. *Arch Physiol Biochem* 2008;114(3):183-94.
29. Miyazaki Y, Pipek R, Mandarino LJ, DeFronzo RA. Tumor necrosis factor alpha and insulin resistance in obese type 2 diabetic patients. *Int J Obes Relat Metab Disord* 2003;27(1):88-94.
30. Bodnar LM, Krohn MA, Simhan HN. Maternal vitamin D deficiency is associated with bacterial vaginosis in the first trimester of pregnancy. *J Nutr* 2009;139(6):1157-61.
31. Lim JH, Ravikumar S, Wang YM, Thamboo TP, Ong L, Chen J, et al. Bimodal Influence of Vitamin D in Host Response to Systemic Candida Infection-Vitamin D Dose Matters. *J Infect Dis* 2015;212(4):635-44.
32. Noyola-Martinez N, Diaz L, Avila E, Halhali A, Larrea F, Barrera D. Calcitriol downregulates TNF-alpha and IL-6 expression in cultured placental cells from preeclamptic women. *Cytokine* 2013;61(1):245-50.
33. Diaz L, Noyola-Martinez N, Barrera D, Hernandez G, Avila E, Halhali A, et al. Calcitriol inhibits TNF-alpha-induced inflammatory cytokines in human trophoblasts. *J Reprod Immunol* 2009;81(1):17-24.
34. Hollis BW, Johnson D, Hulsey TC, Ebeling M, Wagner CL. Vitamin D supplementation during pregnancy: double-blind, randomized clinical trial of safety and effectiveness. *J Bone Miner Res* 2011;26(10):2341-57.
35. Zerofsky MS, Jacoby BN, Pedersen TL, Stephensen CB. Daily Cholecalciferol Supplementation during Pregnancy Alters Markers of Regulatory Immunity, Inflammation, and Clinical Outcomes in a Randomized Controlled Trial. *J Nutr* 2016.

36. Aaltonen R, Heikkinen T, Hakala K, Laine K, Alanen A. Transfer of proinflammatory cytokines across term placenta. *Obstet Gynecol* 2005;106(4):802-7.
37. Zaretsky MV, Alexander JM, Byrd W, Bawdon RE. Transfer of inflammatory cytokines across the placenta. *Obstet Gynecol* 2004;103(3):546-50.
38. Dahlgren J, Samuelsson AM, Jansson T, Holmang A. Interleukin-6 in the maternal circulation reaches the rat fetus in mid-gestation. *Pediatr Res* 2006;60(2):147-51.
39. Much DH, Yeh SY. Prevalence of Chlamydia trachomatis infection in pregnant patients. *Public Health Rep* 1991;106(5):490-3.
40. Pal S, de la Maza LM. Mechanism of T-cell mediated protection in newborn mice against a Chlamydia infection. *Microbes Infect* 2013;15(8-9):607-14.

CHAPTER 4

Vitamin D Mediates the Relationship between Placental Cathelicidin and Group B Streptococcus Colonization during Pregnancy

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ABSTRACT

Vitamin D is thought to modulate innate immune responses, and recent studies have highlighted the autocrine and paracrine functions of vitamin D in the placenta. Our objective was to determine the relationship between maternal vitamin D status and placental antimicrobial peptide (AMP) expression in a group of racially and ethnically diverse pregnant adolescents. In this cross-sectional study, 158 pregnant adolescents were recruited from the Rochester Adolescent Maternity Program (RAMP) in Rochester, NY. Maternal serum concentrations of the vitamin D biomarkers, 25-hydroxyvitamin D (25(OH)D) and 1,25-dihydroxyvitamin D (1,25(OH)₂D), were measured at mid-gestation (~26 weeks) and at delivery. At the placental level, vitamin D regulatory proteins (cubilin, megalin, 1 α -hydroxylase (CYP27B1), 24-hydroxylase (CYP24A1), vitamin D receptor (VDR)) and AMPs (cathelicidin and hepcidin) were analyzed using quantitative PCR and western blot techniques. Placental CYP27B1 mRNA expression was significantly positively associated with both placental cathelicidin mRNA expression ($P < 0.0001$) and placental hepcidin mRNA expression ($P = 0.002$). In teens with positive recto-vaginal group B streptococcus (GBS) colonization, placental mRNA expression of cathelicidin ($P = 0.007$), cubilin ($P = 0.03$), and CYP27B1 ($P = 0.04$) were significantly lower compared to those who tested negative for this infection. A mediation analysis showed that the indirect relationship between GBS colonization and placental cathelicidin mRNA expression was mediated by the placental mRNA expression of the vitamin D proteins cubilin and CYP27B1 ($P = 0.02$). Additional research is needed to identify the role and relative contributions of placental and systemic vitamin D metabolites in relation to potentially pathogenic microorganisms which may be present during pregnancy.

INTRODUCTION

Endogenously synthesized antimicrobial peptides (AMPs) are located on mucosal and epithelial surfaces of multicellular organisms. In innate immunity, these peptides function to kill microorganisms and their activity can be enhanced through synergistic interactions between individual peptides (1) and lysozymes.(2) *In vivo* mouse models have shown increased susceptibility to bacterial infection in AMP gene knockout animals.(3)

Human cathelicidin is a major AMP known to kill a broad range of pathogens, including gram-positive and gram-negative bacteria, fungi, and viruses.(4) In addition, hepcidin is a well-known AMP which functions to limit extracellular microbial growth by preventing the release of intracellular iron, the most essential growth-limiting nutrient for potential pathogens.(5, 6) Both cathelicidin and hepcidin have been shown to be expressed in the human placenta (7, 8), indicating their potential role in mediating maternal infections and preventing pregnancy complications.

Vitamin D status may regulate AMP production given that vitamin D response elements (VDREs) have been identified in the promoter regions of the genes for both cathelicidin and hepcidin.(9, 10) Numerous *in vitro* studies have shown that calcitriol increases the production of the antimicrobial peptide cathelicidin by binding to the VDR in macrophages (9, 11) and *in vitro* studies have shown that low serum 25-hydroxyvitamin D (25(OH)D) levels are associated with reduced production of cathelicidin by human macrophages in response to *Mycobacterium tuberculosis*.(12)

To date, potential relationships between maternal vitamin D status, placental expression of cathelicidin and hepcidin, and clinically diagnosed infections across gestation are poorly described. Therefore, the goal of this study was to 1) elucidate factors associated with placental

AMP expression and 2) to evaluate the degree to which vitamin D has an impact on placental AMP expression at term in a group of high-risk, pregnant adolescents known to have both a high infection burden and suboptimal 25(OH)D status across gestation.

MATERIALS AND METHODS

Study Population

Pregnant adolescents (n= 158, ≤ 18 years old) were recruited from the Rochester Adolescent Maternity Program (RAMP) in Rochester, New York for two larger prospective studies designed to examine changes in maternal and fetal bone health and iron status across gestation (**Table 4.1**). Adolescents between 12 and 30 weeks gestation at entry, carrying a single fetus, and otherwise healthy were eligible to participate. Additional eligibility and exclusion criteria have been previously reported.(13) Informed written consent was obtained from all participants and in participants 14 y of age and younger, adolescent assent and parental consent was also obtained. The study was approved by the Institutional Review Boards at Cornell University and the University of Rochester. Data on recto-vaginal GBS were abstracted from the participant's prenatal medical records as previously documented.(14)

TABLE 4.1: Subject Characteristics of the 158 Pregnant Adolescents¹

Characteristics	Value	Range
Age at enrollment, years (n)	17.1 ± 1.1 (158)	13.6 – 18.7
Maternal Race, % (n)		
African American	63.3 (158)	
Caucasian	35.4 (158)	
Native American	1.3 (158)	
Ethnicity, % (n)		
Hispanic	24.7 (158)	
Non-Hispanic	75.3 (158)	
GA at prenatal care entry, weeks (n)	10.5 ± 4.7 (137)	2 – 24
GA at delivery, weeks (n)	39.2 ± 3.0 (154)	21 – 42.1
Pre-pregnancy BMI, kg/m ² (n)	24.7 ± 5.4 (156)	15.0 – 42.1
Mode of Delivery		
C-Section, % (n)	12 (150)	
Vaginal, % (n)	88 (150)	
Placenta weight, g (n)	606 ± 126 (111)	341 – 979
Placenta area, in ² (n)	153 ± 32.7 (75)	86.4 – 354
Preterm Birth < 37 weeks, % (n)	9.1 (154)	
Mid-gestation 25(OH)D, ng/ml (n)	22.4 ± 10.2 (155)	5 – 61
Mid-gestation 1,25(OH) ₂ D, pg/ml (n)	117 ± 30.3 (100)	46 – 197
Mid-gestation iPTH, pg/ml (n)	28.7 ± 16.0	5 – 97

¹Values are presented as the mean ± SD (n) or % (n); gestational age, GA; body-mass-index, BMI; 25-hydroxyvitamin D, 25(OH)D; 1,25-dihydroxyvitamin D, 1,25(OH)₂D; intact parathyroid hormone, iPTH

Serum Biochemical Analyses

A non-fasting blood sample (10 mL) was obtained at mid-gestation (average: 26 ± 3.4 weeks) (n=155) and at delivery (average: 39.8 ± 1.3 weeks) (n=134). Variability in sample size for the biochemical markers analyzed in this study was due to missed sample collections or insufficient sample volume. Serum samples were stored at –80°C until analysis. The serum vitamin D status biomarker 25(OH)D was measured using a radioimmunoassay (RIA) (Diasorin Inc.), intact parathyroid hormone (iPTH) was measured by enzyme-linked immunoabsorbent assay (ELISA) (Diagnostic Systems Laboratories), and calcitriol (1,25(OH)₂D) was measured by Dr. Michael Holick (Boston, MA) using an in-house thymus receptor binding assay. Data on

vitamin D and calcitropic hormones across gestation in this cohort have been published.(13)

Placental Sample Collection

Placentas were collected at delivery and representative samples of placental tissue were collected as previously described.(15) Representative aliquots of the tissue mixture were placed into RNeasy lysis buffer (Qiagen) or flash frozen (for western blot analyses). All tissue samples were stored at -80°C until analysis.

Quantitative Real-time PCR (qRT-PCR)

Placental RNA samples were prepared and quantitative real-time PCR (qRT-PCR) analyses performed for CYP27B1 and CYP24A1 as previously described.(16, 17) A subset of placental tissue samples (n=86) with quality RNA available was used to conduct new qRT-PCR analyses for hepcidin (*HAMP*), cubilin, megalin, VDR and cathelicidin (*CAMP*) genes using methods as previously detailed.(17, 18) Primers were designed for all targets using the National Center for Biotechnology Information (NCBI) sequence identification numbers and commercially purchased (IDT) as shown in **Table 4.2**. A negative reverse-transcriptase (RT) and template-free (water) sample was used on all plates. Data were normalized to β -actin. All PCR plates also contained a placental tissue control to correct for plate-to-plate variation, and relative mRNA expression was analyzed in relation to a control placenta by calculating $\Delta\Delta$ cycle threshold (CT) values.

TABLE 4.2: Primer Sequences for Target Genes¹

Target	NCBI Sequence No.	Primer Sequence
Cathelicidin (<i>CAMP</i>)	NM_004345.4	Forward: 5'-TGCGCCTGGTGATGCCT-3' Reverse: 5'-CGAAGCACAGCTTCC -3'
Hepcidin (<i>HAMP</i>)	NM_021175.3	Forward: 5'-CTGTTTTCCCACAACAGACG-3' Reverse: 5'-TTCGCCTCTGGAACATGG-3'
CYP27B1	NM_000785.3	Forward: 5'-CACCCGACACGGAGACCTT-3' Reverse: 5'-TCAACAGCGTGGACACAAACA-3'
CYP24A1	NM_000782.4	Forward: 5'-CATCATGGCCATCAAAACAAT-3' Reverse: 5'-GCAGCTCGACTGGAGTGA-3'
VDR	NM_000376.2	Forward: 5'-ACATCGGCATGATGAAGGA-3' Reverse: 5'-TTCCGCTTCAGGATCATCTC-3'
Megalin	NM_004525.2	Forward: 5'-TTGTTTTGATGCCTCTGATGA-3' Reverse: 5'-AGCTAGGCATGTTCGCTCAG-3'
Cubilin	NM_001081.3	Forward: 5'-CCATGGCATCTGTGAGGATT-3' Reverse: 5'-ACAGACGCAGCTGTACTTGG-3'
β -actin	NM_001101.3	Forward: 5'-CCAACCGCGAGAAGATGA-3' Reverse: 5'-CCAGAGGCGTACAGGGATAG-3'

¹National Center for Biotechnology Information, NCBI; vitamin D receptor, VDR.

Western Blotting

Placental protein lysates were prepared and western blot analyses performed for CYP27B1, CYP24A1, and VDR as previously described.(17) For LL-37, protein lysates were diluted in a tris-tricine sample buffer (Bio-Rad). Protein samples (37 μ g) were boiled and separated on a 16.5% tris-tricine pre-cast gel (Biorad) followed by transfer onto Odyssey nitrocellulose membranes (Li-Cor). Membranes were blocked in Odyssey tris-buffered saline (TBS) blocking buffer (Li-Cor). The primary antibody mixture contained 0.2% Tween (Biorad), rabbit (polyclonal) anti-LL-37 (1:1000, Innovagen), and the loading control mouse (monoclonal) anti- β -actin (1:10000, Santa Cruz Biotechnology). Membranes were probed using fluorescent secondary antibodies (LiCor) and protein bands were quantified using the Odyssey IR imaging system (Li-Cor). Similar to other researchers, we detected several bands representing the full length pro-peptide (18 kD)(19, 20), partial processing peptide (14 kD, 12 kD, and 10 kD)(21)

and the active peptide (~6 kD)(20, 21), respectively (**Figure 4.1**). A ratio of the active peptide (~6 kD) to the full length pro-peptide (18 kD) was used for data analysis purposes as this ratio provided more statistically significant results when compared to findings using the active peptide or pro-peptide alone.

FIGURE 4.1

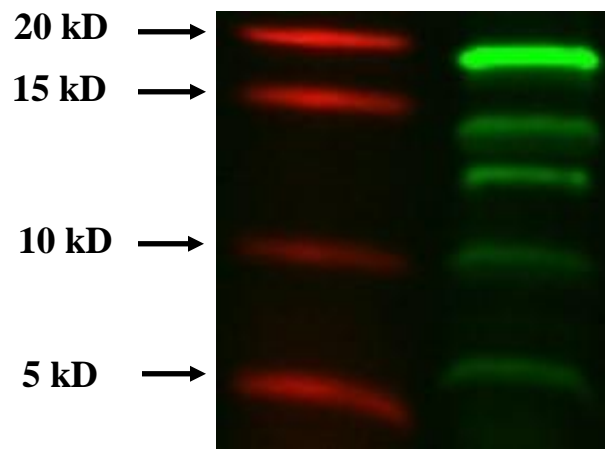


FIGURE 4.1: This is a representative western blot of the ladder (lane 1) and a placental study sample (lane 2). Bands of interest are less than 20 kD. The un-cleaved, pro-peptide (18 kD), partially-processed peptide (14 kD, 12 kD, and 10 kD), and active peptide (~6 kD) were detected.

Statistical Analyses

Simple linear regression was used to examine relationships between placental AMPs (cathelicidin mRNA (*CAMP*), cathelicidin protein (LL-37), and hepcidin mRNA (*HAMP*)), proteins involved in placental vitamin D transport (megalin mRNA, and cubilin mRNA), vitamin D hydroxylases (CYP27B1 mRNA, CYP27B1 protein, CYP24A1 mRNA, CYP24A1 protein) and the vitamin D receptor (VDR mRNA and VDR protein) with circulating serum markers of vitamin D status (25(OH)D and 1,25(OH)₂D). A one-way analysis of variance (ANOVA) was used to analyze differences in mRNA expression and protein abundance of placental AMPs and

placental vitamin D proteins in teens testing positive or negative for recto-vaginal group B streptococcus (GBS). Multiple linear regression analysis was used to assess the relationship between vitamin D and placental AMPs while controlling for potential covariates (maternal race, ethnicity, gestational age at delivery, placental weight, placental area, and mode of delivery). A structural equation model (SEM) was used to identify the direct and indirect relationships between placental vitamin D proteins, recto-vaginal GBS, and placental antimicrobial peptide mRNA expression. Natural log (Ln) transformations were applied to variables which were non-normally distributed. Results were considered statistically significant at $P < 0.05$. All analyses were performed using JMP 12.0 (SAS Institute) and STATA 14.

RESULTS

Subject Characteristics

Subject characteristics are presented in **Table 4.1**. Almost two-thirds of the teens self-identified as African American and one-quarter self-identified as Hispanic. The infection burden in this population was higher than national rates among adolescents for three sexually-transmitted infections (STIs): gonorrhea, chlamydia and trichomoniasis.(14) Rates were also higher compared to national estimates, in pregnant women for recto-vaginal group B streptococcus (GBS), and in the general female population for urinary tract infections (UTIs) and bacterial vaginosis (BV) as previously described.(14)

Mean serum concentrations of vitamin D status biomarkers across gestation in this population have been reported.(13) Using IOM guidelines, 10% of teens were at risk of vitamin D deficiency ($25(\text{OH})\text{D} < 12 \text{ ng/ml}$). Additionally, approximately 50% of the teens and their neonates at birth did not achieve vitamin D sufficiency ($25(\text{OH})\text{D} > 20 \text{ ng/ml}$). (13) However,

using the Endocrine Society's cutoff for vitamin D sufficiency ($25(\text{OH})\text{D} > 30 \text{ ng/ml}$), 81% of adolescents in our study cohort and their neonates at birth did not achieve vitamin D sufficiency.

Circulating Vitamin D and Expression of Placental Vitamin D Trafficking Proteins

The relationships between circulating concentrations of $25(\text{OH})\text{D}$, $1,25(\text{OH})_2\text{D}$, and iPTH and placental vitamin D transport proteins (cubilin and megalin) were explored. Placental megalin and cubilin mRNA expression were both significantly inversely associated with $1,25(\text{OH})_2\text{D}$ at delivery ($r = -0.32$, $n=55$, $P=0.02$ and $r = -0.34$, $n=55$, $P=0.01$, respectively). However, no significant associations between the maternal concentrations of the vitamin D prohormone $25(\text{OH})\text{D}$ or iPTH and placental D transport proteins were evident.

Vitamin D and Maternal GBS

Positive diagnosis of GBS colonization ($n=29$) was associated with significantly lower placental CYP27B1 mRNA expression ($P=0.04$) (**Figure 4.2A**). In addition, significantly lower placental cubilin mRNA expression ($P=0.03$) was evident in those with positive GBS colonization ($n=32$) compared to those who tested negative. In addition, a negative trend was evident between placental VDR protein abundance and positive diagnosis of GBS colonization ($n=38$, $P=0.05$).

FIGURE 4.2

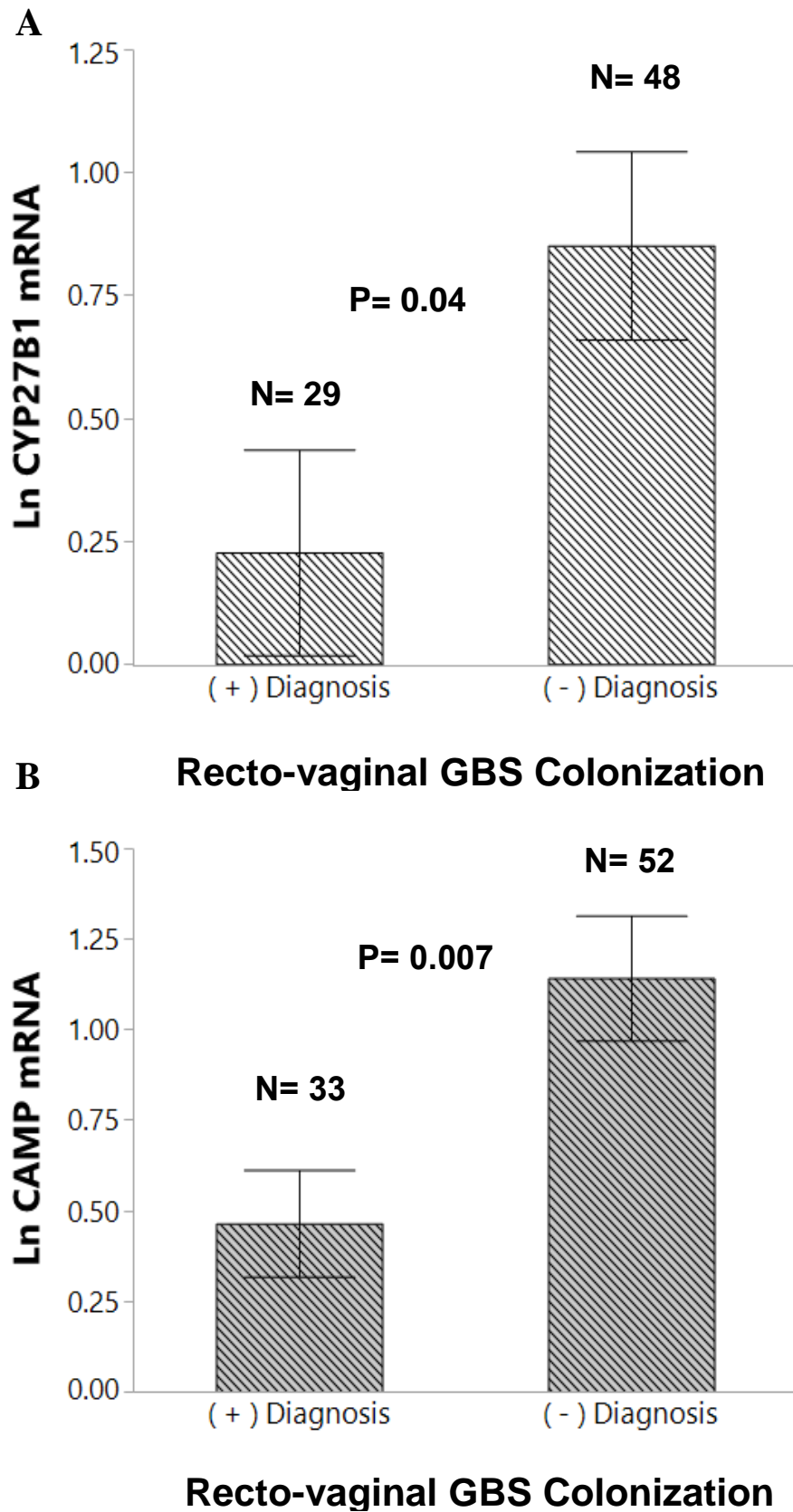


FIGURE 4.2 Positive Diagnosis of recto-vaginal group B streptococcus (GBS) colonization was significantly inversely associated with (A) placental CYP27B1 mRNA expression and (B) placental cathelicidin (*CAMP*) mRNA expression. Standard error bars are displayed.

Placental Antimicrobial Peptides (AMPs)

Significant correlations between placental AMPs were noted. *CAMP* mRNA was significantly positively associated with placental LL-37 protein ($r=0.30$, $n=69$, $P=0.01$) and *HAMP* mRNA ($r=0.33$, $n=86$, $P=0.002$), respectively. The placental mRNA expression of *CAMP* and *HAMP* as well as the protein abundance of LL-37 did not significantly differ as a function of ethnicity, gestational age at delivery, mode of delivery, placental weight, or placental area. However, maternal race was a significant predictor of placental cathelicidin. African American adolescents had significantly lower *CAMP* mRNA expression ($P=0.03$) and LL-37 protein abundance ($P=0.003$) compared to the Caucasian adolescents. After controlling for race, the association between *CAMP* mRNA and LL-37 protein became less significant ($P=0.08$).

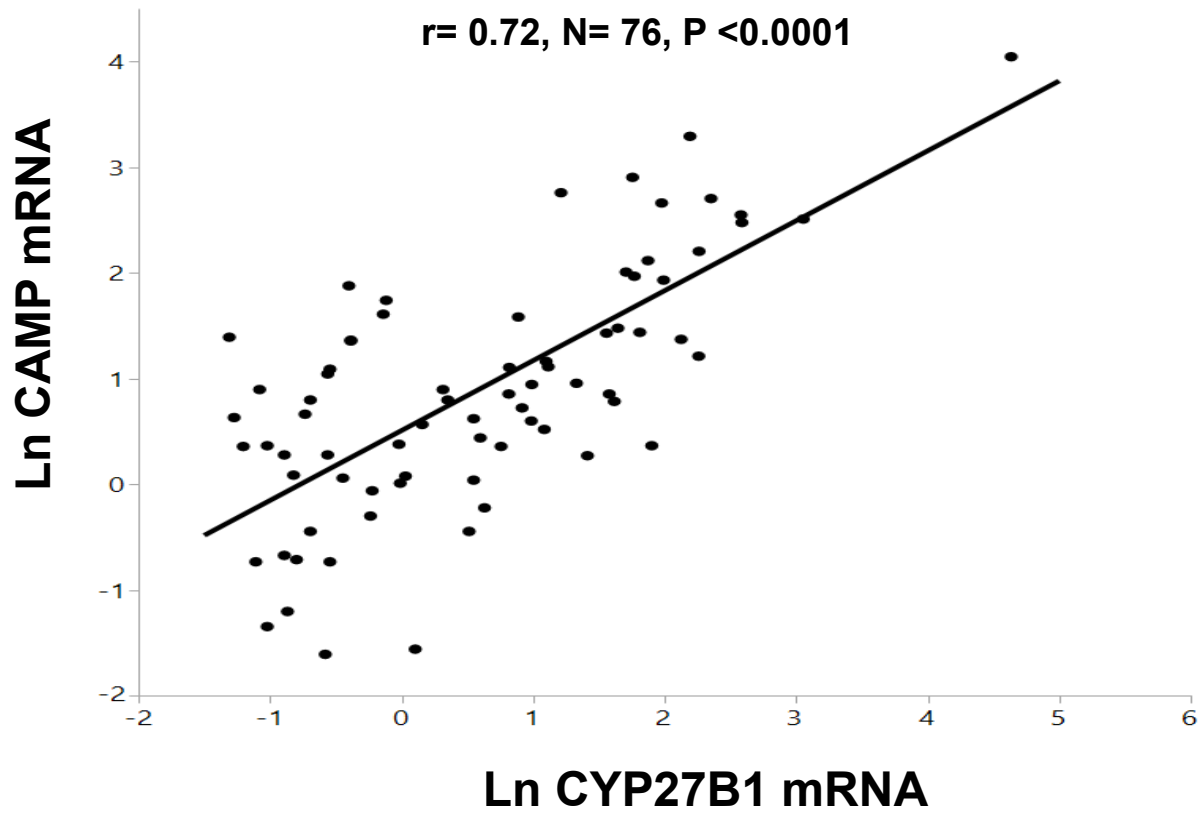
Placental AMPs and Vitamin D

Possible associations between placental AMPs and maternal and neonatal vitamin D status were explored. A significant positive relationship was evident between *CAMP* mRNA and mid-gestation serum $1,25(\text{OH})_2\text{D}$ ($r=0.28$, $n=61$, $P=0.03$) and a positive trend was observed between *HAMP* mRNA and mid-gestation $1,25(\text{OH})_2\text{D}$ ($r=0.25$, $n=61$, $P=0.05$). However, no significant associations between $1,25(\text{OH})_2\text{D}$ and placental AMPs were evident at term. None of the other maternal vitamin D metabolites ($25(\text{OH})\text{D}$ and iPTH) were found to be significantly associated with placental AMPs.

At the level of the placenta, placental CYP27B1 mRNA expression was significantly positively correlated with the placental mRNA expression of *CAMP* ($P < 0.0001$) and *HAMP* ($P = 0.002$) (**Figures 4.3 A and B**). A positive trend was observed between CYP27B1 protein abundance and *HAMP* mRNA expression ($r = 0.21$, $n = 72$, $P = 0.08$). Placental VDR protein abundance was significantly inversely associated with *CAMP* mRNA expression ($r = -0.23$, $n = 83$, $P = 0.04$). *CAMP* mRNA expression was significantly positively correlated with cubilin mRNA expression ($r = 0.32$, $n = 83$, $P = 0.004$).

FIGURE 4.3

A



B

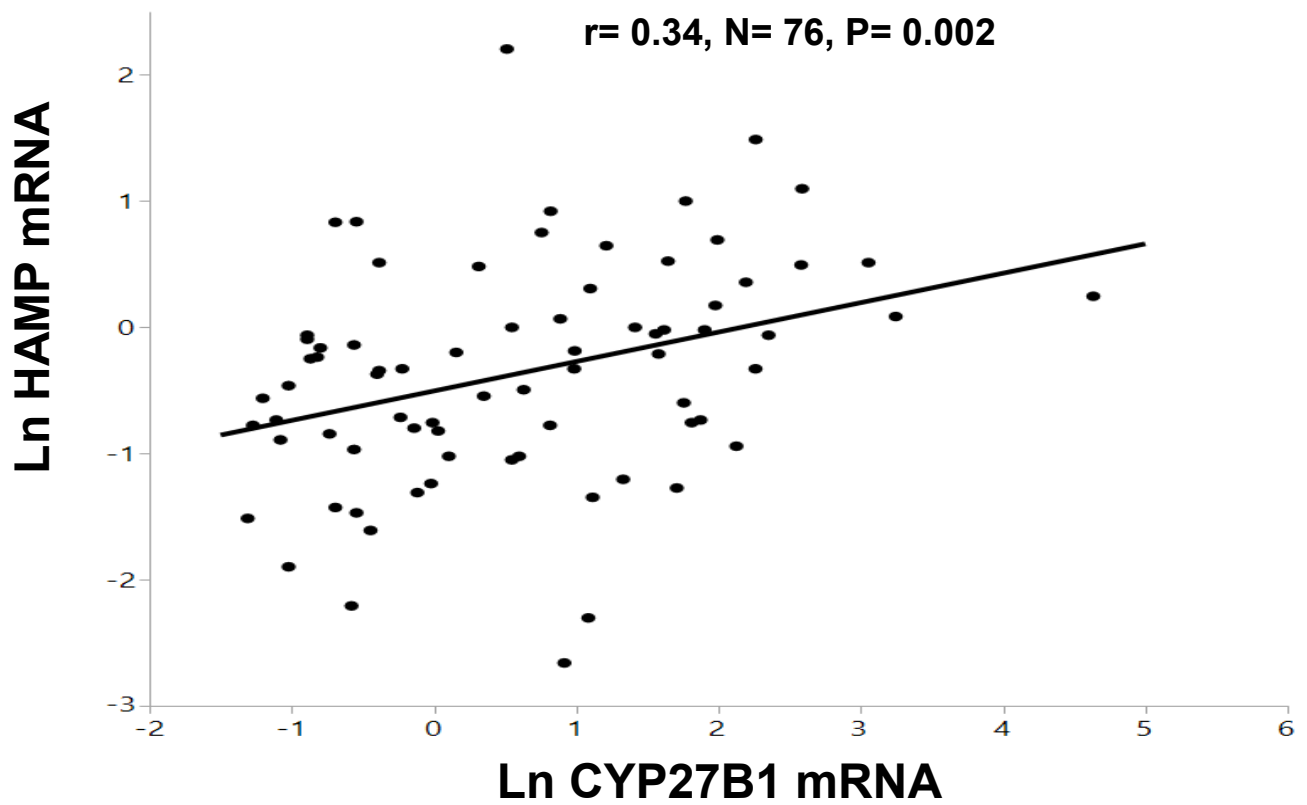


FIGURE 4.3 Placental CYP27B1 mRNA expression is significantly positively associated with (A) placental cathelicidin (*CAMP*) mRNA expression and (B) placental hepcidin (*HAMP*) mRNA expression.

Maternal GBS and Placental AMPs

Associations between maternal infections and placental AMPs were evident. Recto-vaginal group B streptococcus (GBS) colonization was present in 38% of the study population. Compared to those who tested negative for GBS colonization, adolescents with positive diagnosis of GBS colonization showed significantly lower ($P=0.007$) placental *CAMP* mRNA expression (**Figure 4.2B**) but no significant associations were evident between GBS colonization and *HAMP* mRNA expression.

Multivariate Analyses

CYP27B1 mRNA expression remained significantly positively associated with *CAMP* mRNA expression ($P<0.0001$) in a model controlling for maternal race, cubilin mRNA, VDR protein, mid-gestation $1,25(\text{OH})_2\text{D}$, and GBS colonization. To better assess the significant three-way associations between placental *CAMP* mRNA, placental vitamin D (CYP27B1 mRNA and cubilin mRNA), and GBS colonization determined in prior analyses, we conducted an additional mediation analysis (**Figure 4.4**). The direct, indirect, and total effects of GBS colonization on placental vitamin D expression and both GBS colonization and placental vitamin D on *CAMP* expression are displayed in **Table 4.3**.

FIGURE 4.4

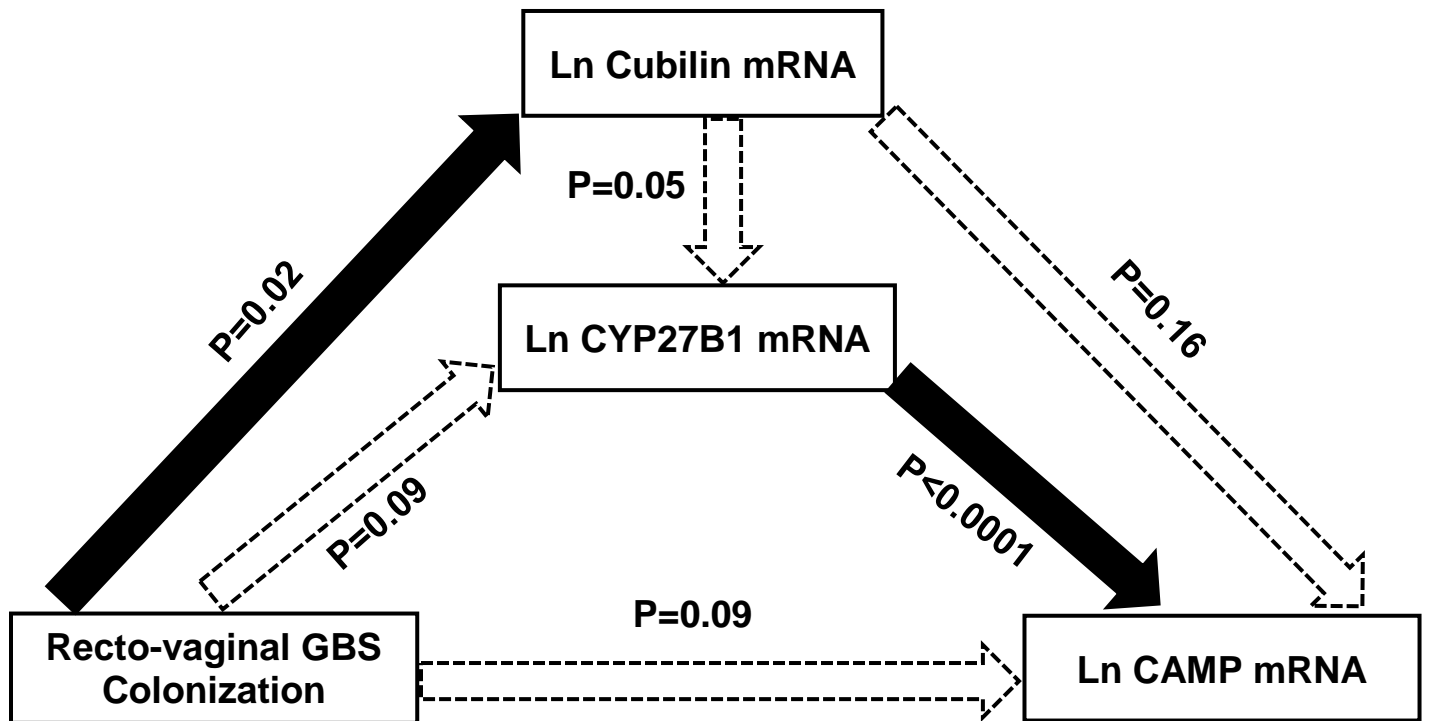


FIGURE 4.4 Diagram displaying the structural equation model (SEM) used to determine the mediating effect of placental mRNA expression of vitamin D transport protein (cubilin) and placental vitamin D metabolism enzyme (CYP27B1) on the association between recto-vaginal group B streptococcus (GBS) colonization and placental cathelicidin (*CAMP*) mRNA expression. Individual direct effects are shown above. Statistically significant associations are represented by solid black arrows.

TABLE 4.3 Mediation Analysis of Placental Vitamin D Expression, Placental Cathelicidin Expression, and Recto-vaginal group B streptococcus (GBS) Colonization in 158 Pregnant Adolescents¹

Proposed Pathway	Indirect & Direct Effects	Total Effects
Cubilin mRNA → <i>CAMP</i> mRNA (direct)	$\beta = 0.12, P = 0.16$	$\beta = 0.27, P = \mathbf{0.02}$
Cubilin mRNA → CYP27B1 → <i>CAMP</i> (indirect)	$\beta = 0.15, P = 0.046$	
GBS Colonization → CYP27B1 mRNA (direct)	$\beta = -0.20, P = 0.09$	$\beta = -0.26, P = \mathbf{0.02}$
GBS Colonization → Cubilin mRNA → CYP27B1 (indirect)	$\beta = -0.06, P = 0.12$	
GBS Colonization → <i>CAMP</i> mRNA (direct)	$\beta = -0.14, P = 0.09$	$\beta = -0.34, P = \mathbf{0.002}$
GBS Colonization → Cubilin + CYP27B1 mRNA → <i>CAMP</i> mRNA (indirect)	$\beta = -0.20, P = \mathbf{0.02}$	

¹Table only includes pathways in which an indirect effect was present. Cathelicidin gene, *CAMP*; group B streptococcus, GBS. Bold-face indicates statistical significance.

DISCUSSION

In this group of 158 pregnant adolescents at risk of vitamin D insufficiency and increased infection burden, maternal serum concentration of the hormonally active form of vitamin D (1,25(OH)₂D) was significantly associated with the mRNA expression of placental vitamin D proteins and placental AMPs. In contrast, no significant associations were evident between the prohormone 25(OH)D and any of the placental AMPs evaluated. Biological effects of calcitriol may be associated with intra-cellular CYP27B1 activity. We found placental CYP27B1 mRNA expression to be positively associated with the expression of two antimicrobial peptides, *CAMP* and *HAMP*. Additionally, lower placental transcript expression of *CAMP* and CYP27B1 was evident in teens testing positive for recto-vaginal group B streptococcus colonization during pregnancy. These results suggest that circulating calcitriol and its placental production may play an important role in mediating transfer risk of maternal-fetal infections during pregnancy.

In this pregnant adolescent population, systemic concentrations of maternal 1,25(OH)₂D measured at delivery were inversely associated with the placental mRNA expression of 25(OH)D transport proteins cubilin and megalin. These findings may be further explained by the fact that

some studies have shown that vitamin D supplementation leads to higher 25(OH)D and calcitriol suggesting that 25(OH)D status constrains 1,25(OH)₂D production in pregnancy.(22) In addition, mid-gestation 1,25(OH)₂D concentrations were previously shown to be significantly positively correlated with placental CYP27B1 mRNA expression in this cohort which may suggest a greater placental hydroxylation of 25(OH)D into calcitriol in teens with elevated systemic calcitriol concentrations.(17) Although maternal serum 25(OH)D concentrations were not associated with the mRNA expression of any of the placental proteins measured (cubilin, megalin, CYP27B1, CYP24A1, or VDR), we previously found that maternal serum 25(OH)D concentrations at mid-gestation and delivery were significantly positively associated with placental CYP27B1 protein expression.(17)

Given the role of 25(OH)D in providing substrate for placental calcitriol production and the ability of circulating calcitriol to impact placental gene expression, both of these metabolites may play important roles in combatting microbial colonization and survival. In this population of pregnant adolescents, recto-vaginal GBS colonization was significantly inversely associated with three placental vitamin D targets (cubilin mRNA, VDR protein, and CYP27B1 mRNA). While we are not aware of other studies looking at placental expression of vitamin D related proteins in relation to GBS, a study in 95 racially-diverse men and women (mean age: 43 years old) reported an inverse association between (serum 25(OH)D concentrations < 30 ng/ml) and increased risk of tuberculosis infection. In vitro studies conducted using primary human macrophages infected with *M. tuberculosis* also found that addition of 1,25(OH)₂D₃ to the culture media significantly reduced the quantity of living bacteria.(12) Prior findings from our adolescent cohort found that teens with lower mid-gestation 25(OH)D status were at greater risk for bacterial vaginosis (BV) and that 1,25(OH)₂D concentrations at delivery were significantly higher in those with positive

bacterial vaginosis infection.(23) While these results suggest that bacterial infections during pregnancy are associated with maternal vitamin D status, supplementation studies are needed to identify causal associations.

Little is known about the relationship between maternal GBS and placental antimicrobial peptides. Our results found a significantly lower placental mRNA expression of the antimicrobial peptide *CAMP* in those testing positive for recto-vaginal group B streptococcus (GBS) colonization compared to those who were negative. These findings may be explained by studies demonstrating bacterial modification of AMPs. Bacteria have been shown to exhibit AMP resistance via bacterial surface modifications (24), AMP degradation via bacterial proteases (25, 26), and AMP sequestering.(27, 28) Downregulation of cathelicidin transcript and peptide has been demonstrated in epithelial-like human cell lines infected with the bacterium *Neisseria gonorrhoeae*.(29) Furthermore, increased bacterial colonization of the urinary bladder mucosa during urinary tract infections has been reported in cathelicidin knockout mice when compared to wild type mice.(30)

Vitamin D may mediate the relationship between GBS during pregnancy and placental AMPs. The mediation analysis demonstrated that the indirect effect of GBS on *CAMP* mRNA expression through cubilin and CYP27B1 mRNA expression explained 59% of the total effect. In this group of pregnant adolescents, a significant positive relationship between mid-gestation serum 1,25(OH)₂D and placental *CAMP* mRNA was evident. Supporting the biological plausibility of our results, 1,25(OH)₂D has been shown to upregulate *CAMP* mRNA in a dose-dependent manner in human monocytes.(12) We observed that placental vitamin D proteins (cubilin mRNA and CYP27B1 mRNA) were also significantly positively associated with placental *CAMP* mRNA expression. Similarly, Liu and colleagues showed that monocytes

cultured in human serum with CYP27B1 antagonists had an 80% reduction in toll-like receptor (TLR) activated *CAMP* mRNA expression.(12) In a small study carried out in 8 European women (median age 62 years old), Hertting and colleagues also found that 12 weeks of daily 2000 IU vitamin D₃ supplementation increased *CAMP* mRNA expression and protein abundance in biopsied urinary bladder tissue infected with *E. coli*.(31)

Racial differences in placental *CAMP* mRNA expression were evident in our study population with significantly lower placental *CAMP* mRNA expression being found among African American adolescents compared to Caucasian adolescents. This finding is consistent with prior data reporting significantly lower *CAMP* mRNA concentrations in monocytes cultured using serum from African Americans.(12) The authors attributed this finding to the lower 25(OH)D serum concentrations that are typically found in African Americans compared to Caucasians, a finding that we documented in this pregnant adolescent cohort.(12, 13)

In addition to cathelicidin, the human placenta also expresses the antimicrobial protein hepcidin. Placental CYP27B1 mRNA expression was positively associated with the mRNA expression of placental antimicrobial *HAMP*, and a positive trend was evident between mid-gestation 1,25(OH)₂D systemic concentrations and *HAMP* mRNA. The regulation of hepcidin in response to circulating or intracellular vitamin D status is not well understood. In contrast to our findings, a study conducted in cultured hepatocytes and monocytes demonstrated reduced hepcidin mRNA following treatment with 25(OH)D or 1,25(OH)₂D. In addition, the same study found a significant reduction in circulating hepcidin concentrations in healthy volunteers (n=7) following a single dose of oral 100,000 IU vitamin D₂.(10) However, the authors suggested that the relationship between vitamin D and hepcidin production may vary depending on the vitamin D status of the population studied.(10) Because many of the adolescents in our study were

vitamin D insufficient, this may explain why the association between $1,25(\text{OH})_2\text{D}$ and hepcidin production was positive in this population.

Additional research on the impact of maternal vitamin D status with intracellular vitamin D proteins, intracellular antimicrobial peptides (AMPs), and infections across pregnancy is needed to better understand biological factors that may affect pregnancy outcomes. Given the role of vitamin D in upregulating *CAMP* expression, our findings suggest that reduced placental vitamin D production may increase susceptibility to infections at the maternal-fetal interface. Vitamin D supplementation studies are needed to evaluate the impact of altered vitamin D status on pregnancy related infections. These data will be useful in identifying possible benefits of maternal vitamin D status on non-calcemic health outcomes. Given that a large percentage of preterm births are thought to be mediated by intra-uterine infections, more research is needed to identify modifiable factors that are associated with decreased risk of infection over gestation.

REFERENCES

1. Hancock RE, Rozek A. Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbiol Lett* 2002;206(2):143-9.
2. Hancock RE, Scott MG. The role of antimicrobial peptides in animal defenses. *Proc Natl Acad Sci U S A* 2000;97(16):8856-61.
3. Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, et al. Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 2001;414(6862):454-7.
4. Lehrer RI, Ganz T. Antimicrobial peptides in mammalian and insect host defence. *Curr Opin Immunol* 1999;11(1):23-7.
5. Drakesmith H, Prentice AM. Hepcidin and the iron-infection axis. *Science* 2012;338(6108):768-72.
6. Armitage AE, Eddowes LA, Gileadi U, Cole S, Spottiswoode N, Selvakumar TA, et al. Hepcidin regulation by innate immune and infectious stimuli. *Blood* 2011;118(15):4129-39.
7. Liu N, Kaplan AT, Low J, Nguyen L, Liu GY, Equils O, et al. Vitamin D induces innate antibacterial responses in human trophoblasts via an intracrine pathway. *Biol Reprod* 2009;80(3):398-406.
8. Best CM, Pressman EK, Cao C, Cooper E, Guillet R, Yost OL, et al. Maternal iron status during pregnancy compared with neonatal iron status better predicts placental iron transporter expression in humans. *FASEB J* 2016;30(10):3541-3550.
9. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, et al. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J Immunol* 2004;173(5):2909-12.

10. Bacchetta J, Zaritsky JJ, Sea JL, Chun RF, Lisse TS, Zavala K, et al. Suppression of iron-regulatory hepcidin by vitamin D. *J Am Soc Nephrol* 2014;25(3):564-72.
11. Gombart AF, Borregaard N, Koeffler HP. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *FASEB J* 2005;19(9):1067-77.
12. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* 2006;311(5768):1770-3.
13. Young BE, McNanley TJ, Cooper EM, McIntyre AW, Witter F, Harris ZL, et al. Vitamin D insufficiency is prevalent and vitamin D is inversely associated with parathyroid hormone and calcitriol in pregnant adolescents. *J Bone Miner Res* 2012;27(1):177-86.
14. Akoh CC, Pressman EK, Cooper E, Queenan RA, Pillittere J, O'Brien KO. Prevalence and Risk Factors for Infections in a Pregnant Adolescent Population. *J Pediatr Adolesc Gynecol* 2016. (In Press)
15. Jaacks LM, Young MF, Essley BV, McNanley TJ, Cooper EM, Pressman EK, et al. Placental expression of the heme transporter, feline leukemia virus subgroup C receptor, is related to maternal iron status in pregnant adolescents. *J Nutr* 2011;141(7):1267-72.
16. Young BE, Cooper EM, McIntyre AW, Kent T, Witter F, Harris ZL, et al. Placental vitamin D receptor (VDR) expression is related to neonatal vitamin D status, placental calcium transfer, and fetal bone length in pregnant adolescents. *FASEB J* 2014;28(5):2029-37.

17. O'Brien KO, Li S, Cao C, Kent T, Young BV, Queenan RA, et al. Placental CYP27B1 and CYP24A1 expression in human placental tissue and their association with maternal and neonatal calcitropic hormones. *J Clin Endocrinol Metab* 2014;99(4):1348-56.
18. Cao C, Pressman EK, Cooper EM, Guillet R, Westerman M, O'Brien KO. Placental heme receptor LRP1 correlates with the heme exporter FLVCR1 and neonatal iron status. *Reproduction* 2014;148(3):295-302.
19. Lim R, Barker G, Lappas M. Human cathelicidin antimicrobial protein 18 (hCAP18/LL-37) is increased in foetal membranes and myometrium after spontaneous labour and delivery. *J Reprod Immunol* 2015;107:31-42.
20. Frew L, Makieva S, McKinlay AT, McHugh BJ, Doust A, Norman JE, et al. Human cathelicidin production by the cervix. *PLoS One* 2014;9(8):e103434.
21. Murakami M, Ohtake T, Dorschner RA, Schitteck B, Garbe C, Gallo RL. Cathelicidin anti-microbial peptide expression in sweat, an innate defense system for the skin. *J Invest Dermatol* 2002;119(5):1090-5.
22. Hollis BW, Johnson D, Hulsey TC, Ebeling M, Wagner CL. Vitamin D supplementation during pregnancy: double-blind, randomized clinical trial of safety and effectiveness. *J Bone Miner Res* 2011;26(10):2341-57.
23. Akoh CC, Pressman EK, Cooper E, Queenan RA, Pillittere J, O'Brien KO. Vitamin D Status is Associated with Infections and Cytokines during Pregnancy. (*Under Review*) 2016.
24. Lysenko ES, Gould J, Bals R, Wilson JM, Weiser JN. Bacterial phosphorylcholine decreases susceptibility to the antimicrobial peptide LL-37/hCAP18 expressed in the upper respiratory tract. *Infect Immun* 2000;68(3):1664-71.

25. McCrudden MT, Orr DF, Yu Y, Coulter WA, Manning G, Irwin CR, et al. LL-37 in periodontal health and disease and its susceptibility to degradation by proteinases present in gingival crevicular fluid. *J Clin Periodontol* 2013;40(10):933-41.
26. Schmidtchen A, Frick IM, Andersson E, Tapper H, Bjorck L. Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Mol Microbiol* 2002;46(1):157-68.
27. Llobet E, Tomas JM, Bengoechea JA. Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. *Microbiology* 2008;154(Pt 12):3877-86.
28. Islam D, Bandholtz L, Nilsson J, Wigzell H, Christensson B, Agerberth B, et al. Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. *Nat Med* 2001;7(2):180-5.
29. Bergman P, Johansson L, Asp V, Plant L, Gudmundsson GH, Jonsson AB, et al. *Neisseria gonorrhoeae* downregulates expression of the human antimicrobial peptide LL-37. *Cell Microbiol* 2005;7(7):1009-17.
30. Chromek M, Slamova Z, Bergman P, Kovacs L, Podracka L, Ehren I, et al. The antimicrobial peptide cathelicidin protects the urinary tract against invasive bacterial infection. *Nat Med* 2006;12(6):636-41.
31. Hertting O, Holm A, Luthje P, Brauner H, Dyrdak R, Jonasson AF, et al. Vitamin D induction of the human antimicrobial Peptide cathelicidin in the urinary bladder. *PLoS One* 2010;5(12):e15580.

CHAPTER 5

CONCLUSION

Key Study Findings and Implications

This doctoral research identified novel findings on risk factors for multiple infections in a pregnant adolescent population and described the relationship between vitamin D, urogenital infections, and biomarkers of potential immunological relevance during pregnancy at both the systemic and placental level. The three specific aims of this dissertation were undertaken and discussed in detail in chapters two through four. In this final chapter, a brief summary of the major findings will be provided, followed by a discussion on the implications of these findings, and suggestions for future research.

In the first specific aim, the prevalence of commonly diagnosed infections in a group of pregnant adolescents was determined. Teens (15-19 y), young adults (20-24 y), and Hispanic and African American minority populations are known to have a high burden of sexually-transmitted infections (STIs), but the prevalence of STIs and other clinically-relevant infections during adolescent pregnancy had not been widely studied. In our pregnant adolescent population, candida (42%), bacterial vaginosis (BV) (40%), recto-vaginal group B streptococcus (GBS) colonization (38%), and cystitis (25%) were the four most prevalent infections/conditions. The prevalence of the latter three infections/conditions in this group of pregnant adolescents was much higher than estimates reported among U.S. adult pregnant populations. Compared to 14-19 year old females in the U.S., the STI burden in our study population was higher for chlamydia, gonorrhea, and trichomoniasis.

Risk factors associated with maternal and placental infections were also

identified in the first study aim. Racial differences were evident such that African American pregnant adolescents had a higher prevalence of BV and at least one of the following STIs: chlamydia, gonorrhea, and trichomoniasis when compared to the Caucasians. Younger maternal age was identified as a risk factor for two STIs: gonorrhea and trichomoniasis. Although older teens and young adults are known to have higher burden of STIs, a possible explanation for the observed results is that pregnant adolescents may engage in higher risk sexual behaviors, particularly in adolescents that become pregnant when they are 15 y of age or younger.

Higher pre-pregnancy body-mass-index (BMI) and low intake of fat soluble vitamins A and D were associated with increased risk for BV, chorioamnionitis, and recto-vaginal GBS, respectively. These findings highlight the importance of modifiable risk factors which may reduce infection risk during pregnancy.

The second research aim was designed to assess correlations between circulating vitamin D and inflammatory biomarkers and to determine the relationship between vitamin D status and clinically-diagnosed urogenital and placental infections during pregnancy. Prior to this research, changes in inflammatory biomarker concentrations with respect to vitamin D status across pregnancy had not been well-studied. The vitamin D prohormone 25(OH)D measured at mid-gestation (~26 weeks) was inversely associated with maternal mid-gestation hepcidin concentrations, but positively correlated with TNF- α at delivery. During pregnancy, the hormonally active vitamin D metabolite, calcitriol, was positively correlated with pro-inflammatory biomarkers (TNF- α , IL-6, and CRP) but at delivery, an inverse association between calcitriol and TNF- α and IL-6 was found. In the neonate, a positive association

between umbilical cord 25(OH)D and TNF- α was evident while an inverse association between cord concentrations of calcitriol and pro-inflammatory biomarkers (hepcidin and IL-6) was observed. These temporal changes in the significant associations found between vitamin D and inflammatory biomarkers highlights the dynamic changes in inflammation and calcitriol that occur across gestation and the need to control for gestational age when examining study findings.

The idea that pregnancy is a state of immune suppression has recently been criticized as an over-simplification. A strong pro-inflammatory response is required in early pregnancy to allow for implantation and placentation, followed by an anti-inflammatory response, and a return to pro-inflammatory state to promote contraction of the cervix during parturition. Because pregnancy is a unique physiological state characterized by immune modulation rather than suppression, the relationship between vitamin D and inflammation would be expected to vary across gestation as the results from our study indicate.

Given that elevated concentrations of pro-inflammatory cytokines have been associated with adverse pregnancy outcomes, such as intra-uterine infections and pre-term birth (< 37 weeks), it is important to understand the biological factors which may contribute to changes in the concentrations of inflammatory biomarkers across gestation. As expected, higher concentrations of maternal and fetal pro-inflammatory biomarkers were evident in those who had a clinically-diagnosed infection during pregnancy. In those testing positive for BV, mid-gestation hepcidin concentrations were significantly higher. Maternal chlamydia infection was associated with higher IL-6 at delivery and higher cord TNF- α and hepcidin concentrations. Placental

chorioamnionitis was associated with increased IL-6 and hepcidin at delivery.

Together, these data indicate that hepcidin may be an additional biomarker of infection during pregnancy and that maternal infections influence fetal inflammatory response.

Lower 25(OH)D status at mid-gestation was individually associated with increased prevalence of two vaginal infections: BV and candida. These results are consistent with other studies conducted in pregnant and non-pregnant adult populations, respectively. Although these findings suggest that low 25(OH)D increases infection risk, an alternative hypothesis proposed by Mangin et al. (122) attributes 25(OH)D depletion and the resulting lower circulating concentrations to increased intracellular calcitriol production during intra-cellular infections.

This study produced a novel finding in that the results demonstrated the relationship between vitamin D and inflammation could be moderated by the presence of an infection. The inverse association observed between calcitriol and TNF- α at delivery was only statistically significant in those who had tested positive for BV at some point across gestation.

In the third study aim, several factors associated with the placental expression of antimicrobial peptides (AMPs) cathelicidin and hepcidin during pregnancy were identified and the impact of vitamin D on AMP expression was determined. In this study, African American pregnant adolescents had significantly lower cathelicidin mRNA expression and protein abundance compared to Caucasian adolescents. This finding is consistent with prior data by Liu et al. reporting significantly lower cathelicidin mRNA concentrations in monocytes cultured in serum obtained from African Americans (110). Consistent with Liu et al.'s findings, we previously

documented that African Americans in our study had lower 25(OH)D concentrations which may explain the racial differences in cathelicidin expression. Although no direct associations between 25(OH)D and placental AMPs were evident, placental CYP27B1 mRNA and circulating calcitriol concentrations were positively associated with placental cathelicidin and hepcidin mRNA.

The placental expression of cathelicidin, cubilin, and CYP27B1 as well as the protein abundance of the vitamin D receptor (VDR) were lower in pregnant adolescents with recto-vaginal GBS colonization. Although, GBS colonization is not thought to have any adverse effects on the mother, transfer of this bacterium to the neonate can cause a systemic neonatal infection (sepsis). A subsequent mediation analysis demonstrated that the relationship between placental cathelicidin expression and GBS was mediated by the placental vitamin D proteins CYP27B1 and cubilin. Our findings suggest that reduced placental vitamin D production may increase susceptibility to infections at the maternal-fetal interface and that vitamin D may be important in regulating placental hepcidin transcript expression at term.

Limitations

There were several limitations in this study which may influence the interpretation of these findings. Although we did detect significant associations between maternal systemic biomarkers (inflammation and vitamin D status), placental biomarkers (AMPs and vitamin D proteins) and infection status, our retrospective cross-sectional study design did not allow us to establish causality between vitamin D, inflammation, and diagnosed infections. We lacked data on whether the study

participants had these infections prior to becoming pregnant as well as if the infection was cleared by antibiotic treatment during pregnancy in those that were diagnosed with repeat infections. It was also unclear how long the infection had been present prior to diagnosis. Data were obtained on systemic cytokine concentrations and site specific concentrations of cytokines were not obtained (such as from the cervix as prior data have found these site specific concentrations of cytokines to be significantly associated with STIs and other vaginal infections). We did not obtain a blood draw during early pregnancy (< 12 weeks) which would have allowed us to better characterize changes in inflammatory biomarkers across pregnancy in relation to vitamin D status. Finally, limitations in sample size may have reduced our ability to detect more significant associations. For example, as expected the prevalence of many of the STIs in the study population was below 10%.

Future Directions

Data from this research provides further insight into a unique and understudied population: racially and ethnically-diverse pregnant adolescents who are at high-risk for vitamin D insufficiency, sexually-transmitted infections, and adverse birth outcomes. These findings highlight the need for continued research on the intersection between vitamin D, inflammation, and infection risk during pregnancy.

This research determined that lower vitamin D status during pregnancy was associated with an increased prevalence of a bacterial and fungal infection. Despite the known impact of CYP27B1 and VDR in the presence of *M. tuberculosis*, the activity of various vitamin D proteins DBP, CYP27B1, CYP24A1, and VDR during other

infections is unclear. Therefore, scientific research would greatly benefit from further studies using animal and cell culture models that are designed to elucidate the underlying molecular mechanisms responsible for the potential impact of vitamin D in reducing infection risk. Because past studies by Aslan et al. and Han et al. have indicated that polymorphisms in VDR are linked to increased infection and disease susceptibility (50, 51), additional human studies exploring genetic polymorphisms in various vitamin D proteins involved in vitamin D metabolism and function during pregnancy are needed.

Although associations between vitamin D and the placental expression of the antimicrobial peptides cathelicidin and hepcidin were identified, additional research should be conducted to determine whether this relationship is found for other vitamin D-responsive antimicrobial peptides, such as β -defensin-2 during healthy and diseased states.

Because this was an observational study, further insight on variable doses of vitamin D and their impact on the incidence, severity, or re-occurrence of infections is needed. Although Zerofsky et al. recently demonstrated that supplementation with 2000 IU of vitamin D₃ at 36 weeks gestation increased the percentage of IL-10+ regulatory CD4+ T cells in peripheral blood greater than that observed following supplementation with 400 IU of vitamin D₃/day (147), the effect of vitamin D supplementation on the concentrations of pro-inflammatory and additional anti-inflammatory biomarkers across gestation is warranted.

Inadequate vitamin A intake was associated with placental chorioamnionitis in this group of pregnant adolescents. Because of the essential role of vitamin A (via

RXR) in enabling VDR function, research should be undertaken to determine the impact of both of these fat-soluble vitamins, in reducing infection and inflammation during pregnancy.

Finally, further research is needed to understand how culturally- and age-appropriate educational resources can be developed to reduce the burden of sexually-transmitted infections among younger, African American adolescents in order to improve maternal health and optimize birth outcomes in this pediatric population.

APPENDICES

Appendix 1: Health Survey Questionnaire

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Maternal / Fetal Bone Health in Pregnant Adolescents Teen Bone Study - Health Survey Questionnaire

Demographics	
MRN: <input type="text"/> <input type="text"/> <input type="text"/> - <input type="text"/> <input type="text"/> - <input type="text"/> <input type="text"/>	DOB: <input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> mm/dd/yyyy
Gestational Age: <input type="text"/> <input type="text"/> weeks* as of	<input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> mm/dd/yyyy*
When was the first day of your last period?	<input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> mm/dd/yyyy*
When is your baby due?	<input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> mm/dd/yyyy*
During what month / week of this pregnancy did you first seek prenatal care? <input type="text"/> <input type="text"/> Month OR <input type="text"/> <input type="text"/> Week	
What was your average weight before pregnancy? <input type="text"/> <input type="text"/> <input type="text"/> lbs* OR <input type="text"/> <input type="text"/> <input type="text"/> kgs*	
How tall are you without shoes on? <input type="text"/> ft <input type="text"/> <input type="text"/> inches OR <input type="text"/> <input type="text"/> <input type="text"/> centimeters	
How old were you when you had your first period? <input type="text"/> <input type="text"/> years old	
<p>Is this your first pregnancy?</p> <p><input type="radio"/> Yes</p> <p><input type="radio"/> Don't know / Not sure</p> <div style="border: 1px solid black; padding: 10px; margin-top: 10px;"> <p><input type="radio"/> NO... How many past pregnancies, including this one?** <input type="text"/> <input type="text"/></p> <p>How many children have you given birth to?** <input type="text"/> <input type="text"/></p> <p>How many abortions have you had?** <input type="text"/> <input type="text"/></p> <p>How many miscarriages have you had?** <input type="text"/> <input type="text"/></p> </div>	
Do you intend to breastfeed your child? <input type="radio"/> Yes <input type="radio"/> No <input type="radio"/> Don't know yet/ Not sure	
Were you using birth control? <input type="radio"/> No <input type="radio"/> Yes, using this type: <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	
<p>Have you had any of the following problems currently or in the past?</p> <p><input type="radio"/> Major Injuries <input type="radio"/> Auto Accidents <input type="radio"/> Broken bones <input type="radio"/> Bone disease <input type="radio"/> Joint Disease</p>	
<p>Have you ever had a sexually transmitted disease (STD)?</p> <p><input type="radio"/> No</p> <p><input type="radio"/> Don't know / Not sure</p> <div style="border: 1px solid black; padding: 10px; margin-top: 10px;"> <p><input type="radio"/> Yes, I have . . . (bubble all that apply)</p> <p><input type="radio"/> Chlamydia <input type="radio"/> Bacterial vaginosis <input type="radio"/> Genital herpes <input type="radio"/> Syphilis</p> <p><input type="radio"/> Gonorrhea <input type="radio"/> Genital warts <input type="radio"/> HIV / AIDS <input type="radio"/> Trichomonas</p> <p><input type="radio"/> Other <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/></p> </div>	

*if unknown, code "?" (i.e. 11/??/2006)

**if not applicable, leave empty.

ID#

Visit Date / / mm/dd/yy

Visit ☐ 1 ☐ 2 ☐ 3

02/15/2007
Page 1 of 3



Maternal / Fetal Bone Health In Pregnant Adolescents
Teen Bone Study - Health Survey Questionnaire

Have any of your family members had any of the following conditions? (grandparents, parents, brothers or children)

		Relationship
Diabetes	<input type="radio"/> Yes <input type="radio"/> No	<input type="text"/>
Heart Disease	<input type="radio"/> Yes <input type="radio"/> No	<input type="text"/>
High Blood Pressure	<input type="radio"/> Yes <input type="radio"/> No	<input type="text"/>
Osteoporosis (brittle bones)	<input type="radio"/> Yes <input type="radio"/> No	<input type="text"/>
Other bone / joint disease	<input type="radio"/> Yes <input type="radio"/> No	<input type="text"/>

Medication	Dose	Unit	Frequency	Start Date (mm/dd/yy)	Stop Date (mm/dd/yy)
<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>

☐ Bubble here if there are more medications not listed.

Did you ever drink or currently drink alcohol?

☐ Never

<input type="radio"/> Currently	drink, <input type="text"/> <input type="text"/> Cups	<input type="radio"/> Every day	Since (mm/yyyy) <input type="text"/> / <input type="text"/> / <input type="text"/>
<input type="radio"/> I Used To		<input type="radio"/> Occasionally	Stop Date* (mm/yyyy) <input type="text"/> / <input type="text"/> / <input type="text"/>
		<input type="radio"/> 2 - 3 times a week	
		<input type="radio"/> Once a week	

Did you ever smoke or currently smoke cigarettes?

☐ Never

<input type="radio"/> Currently	smoke <input type="text"/> <input type="text"/> packs	Since (mm/yyyy) <input type="text"/> / <input type="text"/> / <input type="text"/>	Stop Date* (mm/yyyy) <input type="text"/> / <input type="text"/> / <input type="text"/>
<input type="radio"/> I Used To			

Have you ever used and drugs such as marijuana, cocaine, stimulants, sedatives or other illicit drugs?

Drugs:	<input type="radio"/> marijuana	<input type="radio"/> cocaine	<input type="radio"/> stimulants	<input type="radio"/> sedatives	<input type="radio"/> narcotics	<input type="radio"/> diet pills	<input type="radio"/> other
Dosages:	<input type="radio"/> regularly	<input type="radio"/> regularly	<input type="radio"/> regularly	<input type="radio"/> regularly	<input type="radio"/> regularly	<input type="radio"/> regularly	<input type="radio"/> regularly
	<input type="radio"/> occasionally	<input type="radio"/> occasionally	<input type="radio"/> occasionally	<input type="radio"/> occasionally	<input type="radio"/> occasionally	<input type="radio"/> occasionally	<input type="radio"/> occasionally
	<input type="radio"/> unknown	<input type="radio"/> unknown	<input type="radio"/> unknown	<input type="radio"/> unknown	<input type="radio"/> unknown	<input type="radio"/> unknown	<input type="radio"/> unknown
Started* (yyyy)	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
Stopped* (yyyy)	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>

*write "curr" or "___/curr" if drug is still in use. if unknown, code "P" (i.e. ??/200?) if not applicable, leave empty. (i.e no stop date)

ID# Visit Date / / Visit ☐ 1 ☐ 2 ☐ 3

02/15/2007
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General Demographic Information

 Are you currently covered by medical insurance or a health plan? ☐ Yes ☐ No

 If **YES**, which plan?

 Do you participate in the Women, Infants and Children (WIC) program? ☐ Yes ☐ No

 Do you participate in any other public assistance programs? ☐ Yes ☐ No

 If **YES**, which program?

 Do you live alone? ☐ Yes ☐ No

 If **NO**, who do you live with (bubble all that apply)?

- ☐ Mother ☐ Father ☐ Significant Other ☐ Brother(s) ☐ Sister(s) ☐ Aunt / Uncle
☐ Cousin(s) ☐ Friend ☐ Grandparent(s) ☐ Roommate ☐ Other

 What is your current marital status? ☐ Single ☐ Married ☐ Divorced ☐ Widowed

☐ Other:

 What is your ethnicity? ☐ Hispanic ☐ Non-Hispanic

 What is the ethnicity of the biological father of the baby? ☐ Hispanic ☐ Unknown ☐ Non-Hispanic ☐ Abstain

What is your race?

- ☐ American Indian or Alaska Native
☐ Native Hawaiian or Other Pacific Islander
☐ White / Caucasian
☐ Asian
☐ Black or African American ☐ Other

What is the race of the biological father of the baby?

- ☐ American Indian or Alaska Native
☐ Native Hawaiian or Other Pacific Islander
☐ White / Caucasian ☐ Abstain
☐ Asian ☐ Unknown
☐ Black or African American ☐ Other

 What is your highest level of education completed? Years 0-6 = Primary School 7-12 = Secondary

Contact Information

Home Phone Number

 - -

Alternate Phone Number

 - -

Address

Emergency Contact Phone Number

 - -

Relationship

Name

Other
☐ Yes ☐ No Relatives With Patient? Who?

 ID#

 Visit Date
mm/dd/yy

 / /

 Visit ☐ 1 ☐ 2 ☐ 3

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Appendix 2: Baby Data Form

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Teen Bone Study
Baby Data Form

Baby Data									
Delivery Date: <input type="text"/> mm / <input type="text"/> dd / <input type="text"/> yy			Delivery Time: <input type="text"/> : <input type="text"/> <input type="radio"/> AM <input type="radio"/> PM			Sex: <input type="radio"/> Male <input type="radio"/> Female			
Preterm: <input type="radio"/> Yes <input type="radio"/> No									
Attending Provider: <input type="text"/>			Gravida: <input type="text"/>		Para: <input type="text"/>				
GA at Delivery: <input type="text"/> W <input type="text"/> D			Revised EDC: <input type="text"/> / <input type="text"/> / <input type="text"/> mm/dd/yy						
Height: <input type="text"/> in		Prepregnant Wt: <input type="text"/> lbs		Current Wt: <input type="text"/> lbs		Weight gain: <input type="text"/> lbs			
Type of Delivery: <input type="radio"/> SVD <input type="radio"/> Assisted <input type="radio"/> 1st C-section <input type="radio"/> 2nd C-section <input type="radio"/> V-BAC									
Antepartum Complications: <input type="text"/>									
Duration of labor: Stage 1 <input type="text"/> hr <input type="text"/> min Total Labor Time <input type="text"/> hr <input type="text"/> min									
Stage 2 <input type="text"/> hr <input type="text"/> min									
Stage 3 <input type="text"/> hr <input type="text"/> min									
Delivery Complications: <input type="text"/>									
PE/PIH: <input type="radio"/> Yes <input type="radio"/> No									
Weight at birth: <input type="text"/> gms			Length at birth: <input type="text"/> cms			Head circumference: <input type="text"/> cms			
Bubbles: <input type="radio"/> SGA/IUGR <input type="radio"/> LGA									
APGAR scores: 1 min. <input type="text"/> 5 min. <input type="text"/> 10 min. <input type="text"/>									
Presence of meconium: amniotic fluid: <input type="radio"/> Yes <input type="radio"/> No meconium aspiration: <input type="radio"/> Yes <input type="radio"/> No									
Discharged to: <input type="radio"/> Newborn Nursery <input type="radio"/> SC Nursery <input type="radio"/> Strong NICU <input type="radio"/> Other (specify below)									
<input type="text"/>									
Medication		Frequency		Date (mm/dd/yy)					
<input type="radio"/> Mom <input type="radio"/> Baby									
<input type="text"/>		Dose <input type="text"/>		<input type="text"/>		Start <input type="text"/> / <input type="text"/> / <input type="text"/>			
<input type="text"/>		Unit <input type="text"/>		<input type="text"/>		Stop <input type="text"/> / <input type="text"/> / <input type="text"/>			
<input type="radio"/> Mom <input type="radio"/> Baby									
<input type="text"/>		Dose <input type="text"/>		<input type="text"/>		Start <input type="text"/> / <input type="text"/> / <input type="text"/>			
<input type="text"/>		Unit <input type="text"/>		<input type="text"/>		Stop <input type="text"/> / <input type="text"/> / <input type="text"/>			
<input type="radio"/> Mom <input type="radio"/> Baby									
<input type="text"/>		Dose <input type="text"/>		<input type="text"/>		Start <input type="text"/> / <input type="text"/> / <input type="text"/>			
<input type="text"/>		Unit <input type="text"/>		<input type="text"/>		Stop <input type="text"/> / <input type="text"/> / <input type="text"/>			
<input type="radio"/> Mom <input type="radio"/> Baby									
<input type="text"/>		Dose <input type="text"/>		<input type="text"/>		Start <input type="text"/> / <input type="text"/> / <input type="text"/>			
<input type="text"/>		Unit <input type="text"/>		<input type="text"/>		Stop <input type="text"/> / <input type="text"/> / <input type="text"/>			

Office Use Only

ID#

03/05/2009
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Appendix 3: Infections and Inflammatory Conditions Chart

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Teen Vitamin D Study Infections and Inflammatory Conditions

Upper Respiratory Infections				
Asthma	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
	Present	Date noted		
Bronchitis	Yes			
	No			
Treated with:				
Notes				
Pneumonia	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
Ear Infections	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
Sinus Infections	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
Strep Throat	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
General Infections				
Mononucleosis	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
	Present	Date noted		
Scabies	Yes			
	No			
Treated with:				
Notes				

ID #: _____ Visit Date: ____/____/____ Visit #: 1__ 2__ 3__



Teen Vitamin D Study
Infections and Inflammatory Conditions

Page 2 of 4

General Infections (continued)				
Shingles	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
Lice	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
Sexually Transmitted Diseases				
Gonorrhea	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
Bacterial Vaginosis	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
Mononucleosis	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
Chlamydia	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
Syphilis	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
Genital Warts	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes:				

ID #: _____ Visit Date: ____/____/____ Visit #: 1__2__3__

2



Teen Vitamin D Study
Infections and Inflammatory Conditions

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Infections and Inflammatory Diseases				
Genital Herpes	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
Trichomoniasis	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
Candida	Present	Date Noted		
	Yes			
	No			
Treated with:				
Notes				
Placental Infections				
Chorioamnionitis	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
Chronic Vilitis	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
Urinary Tract Infections				
Pyelonephritis	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
Cystitis	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes:				

ID #: _____ Visit Date: ____/____/____ Visit #: 1__ 2__ 3__

3



Teen Vitamin D Study
Infections and Inflammatory Conditions

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Other Infections				
Hepatitis B	Present	Date Noted		
	Yes			
	No			
Treated with:				
Notes				
Other Infections (continued)				
Hepatitis C	Present	Date Noted		
	Yes			
	No			
Treated with:				
Notes				
HPV	Present	Date Noted		
	Yes			
	No			
Treated with:				
Notes				
Other Miscellaneous Infections / Inflammatory Processes				
	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				
	Present	Date noted		
	Yes			
	No			
Treated with:				
Notes				

ID #: _____ Visit Date: ____/____/____ Visit #: 1__ 2__ 3__

4

Appendix 4: Youth Assent, Parent Permission, and Consent

Youth Assent Form

Study Title: **Maternal / Fetal Bone Health in Pregnant Adolescents**

Principal Investigators: **Thomas McNanley, M.D., Elizabeth Cooper, CNM, EdD, FACNM**

Co-Investigator: **Kimberly O'Brien, Ph.D.**

Introduction:

This form describes a research study that we are asking you to be in. You are invited to take part in this study because you are pregnant and 18 years or younger and are healthy. Teens need more calcium in their diet than adult women so that they can build strong healthy bones. Teens that are also pregnant may need even more calcium. We're asking you to help us find out more about calcium and bone health in pregnant teens by being part of this study. Please read this form carefully and ask the person who presents it any questions you may have before you decide whether or not to be in the study.

Dr's Thomas McNanley, Elizabeth Cooper and Kimberly O'Brien at the University of Rochester are doing this study.

Purpose of Study

The purpose of the study is to learn more about bone health in pregnant teens and about how babies' bones grow before they are born.

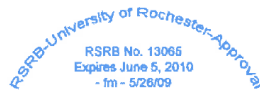
Description of Study Procedures

We hope that you will help us by being in this study, but it is up to you. You don't have to be in this study. If you start it, you can still stop any time you want. If you are in the study for the whole time, from beginning to end, it will take about 9 months to finish.

In this study, at 3 different times over your pregnancy we will:

- Measure how tall you are and how much you weigh
- Ask you some questions about what you usually eat, and how much you exercise and if you use drugs or alcohol
- Take a measurement of your blood pressure and heart rate
- Measure the distance between your knee and heel

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- Take a picture of your baby (called an ultrasound or sonogram)
- Measure how much bone is in your heel (using a special machine that will not hurt you or your baby)

Each visit may take up to two hours, so we will offer you some food and a drink during that time.

At one of these visits in the middle of your pregnancy we will also:

- Take a small blood sample from your arm. Before the blood is taken, the nurse will ask you if you want some special cream to be put on your arm to make it hurt less.

Second Part of Study (Optional)

If you want to, you can also be in the second part of this study on the day your baby is born. It is okay if you do not want to participate in this second part of the study. You can still be in the first part of the study.

If you want to be in the second part, we will:

- Take some blood from your arm.
- See how much your baby weighs and how long he/she is
- After a baby is born, the baby's afterbirth (called the placenta) comes out. The placenta is usually thrown away. Before it is thrown away we will take some blood and pieces of the placenta. This will help us to learn more about how the placenta sends food to your baby when you were pregnant.

We will use the blood sample we get from the placenta to measure:

- Things your baby needs for healthy bones
- Levels of lead in your baby's blood

If your baby has too much lead in their cord blood, we will call the baby's doctor right away. The baby's doctor can decide if your baby needs medicine to get rid of this lead.

We also would like to take a picture of your bones and your baby's bones using a special test called a DXA. We will try to do this test within 4 weeks after your baby is born. You will need to go to Strong Memorial Hospital for this final test.

- The picture of your bones is like an x-ray. It takes about 5-10 minutes to complete. It is safe to use if you are not pregnant.

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You should not have this test if you may be pregnant. Therefore, you will need to read and sign the "DXA waiver" and you may be tested for pregnancy before the DXA using a urine test.

- The picture of your baby's bones will take about 2 minutes to complete. Your baby will be wrapped in a blanket and lie on a padded table for the test. You can stay in the room with your baby while the test is conducted.

To complete the entire visit for this study should take 40-60 minutes.

Risks of Participation

There are no known risks from the pictures we will take of your heel and of your baby. You may get a bruise and it may hurt a bit when the blood samples are taken. Sometimes the cream used to numb your arm may cause irritation. Some people feel dizzy or faint when their blood is drawn.

The picture of your bones and your baby's bones involves an x-ray. The radiation amount is less than a person would receive from a chest x-ray or a dental x-ray or about what you would get in one day from the natural radiation around us. We don't know whether any radiation dose is completely safe. The risk from the dose you and your baby will receive is considered low compared to other everyday risks.

Benefits of Participation

Screening for lead in your blood during pregnancy may be a benefit. High blood levels can be treated. We will tell you and your doctor if we find high lead levels. You may not benefit from being in the study.

Contact Persons

If you have any questions about the study, please call: Beth Cooper in the RAMP clinic at (585) 275-2962 or Kimberly O'Brien at (607) 255-3743.

If you have any questions about your rights as a research subject, you may contact the Human Subjects Protection Specialist at the University of Rochester Research Subjects Review Board, Box 315, 601 Elmwood Avenue, Rochester, NY 14642-8315, Telephone (585) 276-0005, for long-distance you may call toll-free, (877) 449-4441.

Voluntary Participation

Whether or not you take part in this study is up to you. You don't have to take part and you can stop any time, for any reason. If you decide to

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stop, it won't change any other part of your care. If you do decide to stop, we will not tell other people about the information we have collected.

You don't have to take part in this study, even if your parents want you to.

Subject Consent

Second Part of Study

Please check one:

- ☐ I also agree to participate in the second part of the study when my baby is born.
- ☐ I do not agree to participate in the second part of the study when my baby is born.

Future Studies

The investigators from this study may want to contact you in the future regarding this study or to see if you and/or your baby would be interested in participating in future studies. At this time you may decide whether or not you want to be contacted. If and when you are contacted you may decide if you and/or your baby want to participate in any of the other studies and will sign another consent form to participate in those studies. Your decision regarding future contacts will not affect your participation in this study.

Please check one:

- ☐ Yes, I may be contacted in the future regarding this study **or** future studies.
- ☐ Yes, I may be contacted in the future for this study, but **not** for future studies.
- ☐ NO, I may not be contacted in the future.

Blood Samples

If it is okay with you we would also like to collect white blood cells from your blood so that we can screen for DNA and genes involved in how the body uses nutrients. The samples may be used to help identify genetic factors that influence how the body uses nutrients and to understand how

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- 1m - 5/28/09

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these may be related to differences in bone loss or fetal bone growth. The samples will not be sold or used directly for the production of commercial products and will be kept in a locked lab. Reports about future research done with the sample will NOT be kept in your health records, but the sample reports may be kept with study records or in other secure areas.

You can decide if you want your sample to be used for this type of research. Your decision can be changed at any time by notifying the study doctor in writing. Your decision about your sample will not affect your participation in this study or other studies.

Please check one:

- ☐ Yes, you may use my blood sample for the DNA studies described above.
- ☐ NO, you may not use my sample for the DNA studies described above.

Signatures/Dates

I have read this form (or had it read to me). If I had questions, they were answered. I agree to be in this study.

Study Subject: _____ Print Name

Study Subject: _____ Signature

_____ Date

Person Obtaining Assent:

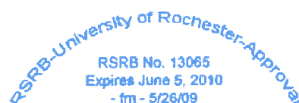
I have read this form to the subject and/or the subject has read this form. An explanation of the research was given and questions from the subject were solicited and answered to the subject's satisfaction. In my judgment, the subject has demonstrated comprehension of the information.

_____ Print Name and Title

_____ Signature

_____ Date

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Parent Permission Form

Study Title: **Maternal / Fetal Bone Health in Pregnant Adolescents**
Principal Investigators: **Thomas McNanley, M.D., Elizabeth Cooper, CNM, EdD, FACNM**
Co-Investigator: **Kimberly O'Brien, Ph.D.**

Introduction:

This consent form describes a research study and what you may expect if your child decides to participate. Please read this form carefully and ask the person who presents it any further questions you may have before making your decision whether or not to participate.

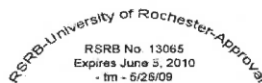
This study is being conducted by Thomas McNanley, MD, Beth Cooper, CNM, EdD and Kimberly O'Brien, PhD, of the University of Rochester's Department of Obstetrics and Gynecology and Highland Hospital's Department of Obstetrics and Gynecology. Kimberly O'Brien is also on the faculty at Cornell University.

Your child is being asked to participate in this study because she is pregnant and 18 years of age or younger.

Purpose of Study

The purpose of the study is to learn more about bone health in pregnant teens and about how babies' bones grow before they are born. Calcium and vitamin D are found in foods that you eat and are needed for healthy bones. Teenage girls build most of their bone by the time they are 17-18 years old. When teenagers become pregnant they may need extra calcium. Too little calcium and vitamin D in the diet might cause teenage girls to lose bone. It might also slow the growth of their baby's bones.

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Your daughter can volunteer for this study if she:

- Is 18 years old or younger
- Is less than 30 weeks pregnant
- Does not have high blood sugar (diabetes)
- Does not have HIV infection
- Does not have problems eating and digesting her food
- Does not have an eating disorder (such as anorexia)

Description of Study Procedures

If your daughter decides to participate in this study she will be asked some questions to see how healthy she is. We will also look in her medical chart to learn more about how healthy she is and to learn more about her pregnancy. We would like her to come to Highland Hospital up to three times during her pregnancy, with visits usually being spaced at least four weeks apart so we can:

- Measure how tall she is and how much she weighs
- Take a measurement of her blood pressure and heart rate
- Measure the distance between her knee and heel
- Take a picture of her baby (called an ultrasound or sonogram). This test is safe to use during pregnancy.
- Ask her questions about what she eats and how she decides what to eat, and about how much she exercises
- Ask her questions about how often she uses drugs or alcohol; and
- Measure how much bone she has in her body by putting her heel in a special ultrasound machine that will not hurt her or her baby.

Each visit may take up to two hours, so we will offer her some food and a drink during that time.

At one of these visits, when she is approximately **20 - 30 weeks pregnant**, we will also:

- Take a blood sample from her arm (about 4 teaspoons). Before the blood is taken, the nurse will ask her if she wants some special cream to be put on her arm to make it hurt less. We will keep this blood sample until the entire sample collected has been used to measure nutrients.

We will use this blood sample to measure:

- Hormones and proteins that her body needs when she is pregnant
- Levels of lead in the blood
- Levels of vitamin D in the blood

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Lead is found in many homes in Rochester. If it gets into your daughter's body it can make your daughter and her baby sick. We will call her doctor right away if she has too much lead in her blood. Her doctor can decide if she needs medicine to get rid of this lead. If she has very high lead in her blood, she will not be able to continue in this study.

Second Part of Study (Optional)

If your daughter wants to, she can also participate in the second part of the study on the day her baby is born. It is okay if she does not want to participate in this second study. She can still be in the first part of the study. If she wants to participate in this second part, her medical chart will have a sticker put on it. The sticker will let the doctors and nurses know that they should call us after she goes into labor and before her baby is born.

If your daughter decides to participate in part 2, we will:

- Collect a sample of her blood (3 teaspoons). A blood sample is normally taken from her arm when she enters the hospital to have her baby and the sample we are collecting will be drawn at the same time. We will use this sample and the blood sample from the placental cord to measure hormones and proteins that her body needs when she is pregnant.
- Record the birth weight and birth length and other health information on her baby from her baby's medical chart.
- After a baby is born, the baby's afterbirth (called the placenta) comes out. The placenta is usually thrown away as waste. Before it is thrown away we will take some pieces of the placenta. This will help us to learn more about how the placenta sends nutrients to the baby while your daughter was pregnant. We will also take some blood from the cord of the placenta (2-3 teaspoons). This cord blood sample will tell us about the nutrients and hormones that your daughter's baby has when he/she is born. We will keep the blood samples until the entire sample has been used to measure nutrients.

We will use the blood sample we get from the placental cord blood to measure:

- Hormones and proteins that the baby needs for healthy bones
- Levels of lead in the blood
- Levels of vitamin D in the blood

If your daughter has lead in her body some of this lead may be sent to her baby while she is pregnant. This lead can make her baby sick. If your daughter's baby has too much lead in their cord blood, we will call the baby's doctor right away. The baby's doctor can decide if your daughter's baby needs medicine to get rid of this lead.

We also would like to measure how much bone your daughter and her baby have in their bodies using a special test called a DXA. The DXA machine is located at Strong Memorial Hospital. We will try to schedule this test within 4 weeks of the baby's delivery and ask your daughter to come to Strong Memorial Hospital with her baby for this final test.

The Dual energy X-ray absorptiometry (DXA) scan measures bone density of your daughter's whole body and of her lower spine. For the whole body scan, she will be asked to lie flat on her back on a table as the scanning machine moves above her body. From the scan of her whole body we will be able to see how much calcium she has in her entire body. We can compare this to the amount that is normally found in teenagers that are her age. This test will also measure the amount of fat and lean tissue in her body. For the scan of her lower back she will be asked to also lie on her back on the padded table, a large fabric covered foam cube will be placed under her lower legs so that she is in the best position to take the measure of the lower back area. These DXA scans are like an X-ray, and these tests will take about 5-10 minutes to complete. It is safe to use if your daughter is not pregnant. She should not have this test if she may be pregnant. Therefore, she will be asked to read and sign the DXA waiver and she may be tested for pregnancy before the DXA using a urine test.

The DXA test on the newborn will take approximately 2 minutes. The baby will be wrapped in a blanket and lie on a padded table for the test. Your daughter can stay in the room with the baby while the test is conducted.

To complete the entire visit for this study should take 40-60 minutes.

Number of Subjects

We plan to recruit a total of 300 adolescents to volunteer for this research study.

Risks of Participation

There are no known risks from the ultrasounds that we will take of your daughter's heel and of her baby. A total of 4 teaspoons of blood will be taken during pregnancy. A total of 7 teaspoons of blood will be taken if she volunteers for both studies. She may get a bruise and it may hurt a bit when the blood samples are taken. Some people feel lightheaded or

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faint when their blood is drawn. There is also a rare risk of infection. The samples of the placenta are collected after the baby is born and will not cause any risk. Use of the cream to numb her arm may cause irritation.

The DXA scans your daughter and her baby will receive if she participates in part 2 of the study (the delivery part) involves radiation exposure which is less than a person would receive from a chest X-ray or a dental X-ray. The amount of radiation from this test is also similar to what we receive from the natural background each day. We don't know whether any radiation dose is completely safe. The risk from the dose your daughter and her baby will receive is considered low compared to other everyday risks.

Benefits of Participation

Screening for lead in your daughter's blood during pregnancy may be a benefit. High blood levels can be treated. We will tell you and her primary care provider if we find high lead levels. We will also test her blood for levels of vitamin D. Low vitamin D can be treated with supplements. If she is found to have low vitamin D levels we will tell you and share this information with her primary care provider. There is no health benefit provided by ultrasound screening. The ultrasound of her baby is only to tell us about the growth of her baby. However, if we found anything that made us worry that her baby was not healthy, we would tell you and her obstetrician right away.

Alternatives to Participation

Your daughter does not have to participate in this study if she does not want to. Her decision not to join this study will not affect the health care she receives at Highland Hospital or elsewhere.

Payments

Part 1:

For completing the **first visit** your daughter will be given;

- A sonogram picture of the baby
- A small photo album

For completing the **second visit** your daughter will be given;

- A sonogram picture of the baby
- A \$10 Target or Walmart gift card (she can decide which store she prefers)

For completing the **third visit** your daughter will be given;

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Part 2:

If your daughter participates in part 2 when her baby is born we will take a digital picture of her with her baby, if she would like. The picture is a gift and will not be saved or used for other research purposes. She can have her picture taken in the hospital or during her post partum visit at RAMP. On the day that she comes into the GCRC at Strong Memorial Hospital for the DXA study she will receive \$25.00 in cash.

Sponsor Support

The University of Rochester is receiving payment from the USDA for conducting this research study.

Confidentiality of Records and HIPAA Authorization

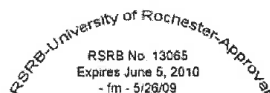
While we will make every effort to keep information we learn about you private, this cannot be guaranteed. Other people may need to see the information. While they normally protect the privacy of the information, they may not be required to do so by law. Results of the research may be presented at meetings or in publications, but your name will not be used.

The federal Health Insurance Portability and Accountability Act (HIPAA) requires us to get your permission to use health information about you that we either create or use as part of the research. This permission is called an Authorization. We will use;

- Demographic information (information on where you live, your phone number, etc.)
- Information on your height, weight and previous pregnancies
- Dietary information and information on supplement use over pregnancy
- Self reported drug and alcohol use and use of cigarettes
- Current use of medications and prescription drugs
- Diagnosis of any pregnancy complications or health problems
- Test results on hemoglobin and routine tests drawn across pregnancy
- The place where you were seen
- The name of your physician
- The medical records of your newborn.

We will use your health information to conduct the study and to determine how your health status and other medical care issues that are happening during your pregnancy might be influencing the health of your bones and the growth of your developing baby. Health information is used to report results of research to sponsors and federal regulators. The health information collected may be audited to make sure we are following regulations, policies and study plans. URM/Strong Health policies let you see and copy health information we have gathered for this research study after the study ends, but not until the study is

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completed. If you have never received a copy of the URM/ Strong Health HIPAA Notice of Privacy Practices, please ask the investigator for one.

To meet regulations or for reasons related to this research, the study investigator may share a copy of this consent form and records that identify you with the following people. The University of Rochester; the Department of Health and Human Services; the United States Department of Agriculture, Cornell University, University of Rochester, Highland Hospital, and your primary care provider.

If you decide to take part, your Authorization for this study will not expire unless you cancel (revoke) it. The information collected during your participation will be kept indefinitely. You can always cancel this Authorization by writing to the study investigator. If you cancel your Authorization, you will also be removed from the study. However, standard medical care and any other benefits to which you are otherwise entitled will not be affected. Canceling your Authorization only affects uses and sharing of information after the study investigator gets your written request. Information gathered before then may need to be used and given to others.

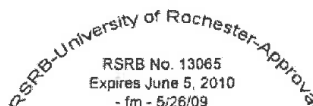
As stated in the section on Voluntary Participation below, you can also refuse to sign this consent/Authorization and not be part of the study. You can also tell us you want to leave the study at any time without canceling the Authorization. By signing this consent form, you give us permission to use and/or share your health information as stated above.

Contact Persons

For more information concerning this research, please contact: Beth Cooper in the RAMP clinic at (585) 275-2962 or Kimberly O'Brien at (607) 255-3743.

If you have any questions about your rights as a research subject, you may contact the Human Subjects Protection Specialist at the University of Rochester Research Subjects Review Board, Box 315, 601 Elmwood Avenue, Rochester, NY 14642-8315, Telephone (585) 276-0005, for long-distance you may call toll-free, (877) 449-4441. You may also contact the Cornell University Committee on Human Subjects (UCHS) at 607-255-5138, or via the web at:
<http://www.osp.cornell.edu/Compliance/UCHS/homepageUSHS.htm>.

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Voluntary Participation

Participation in this study is voluntary. Your child is free not to take part or to stop at any time, for any reason, without losing present or future care he/she would expect to receive. In the event that you do withdraw your child from this study, we will keep the information we have collected confidential.

Parental Permission

Please check one:

- ☐ I also agree to allow my daughter to participate in the second part of the study when her baby is born.
- ☐ I do not agree to allow my daughter to participate in the second part of the study when her baby is born.

Future Studies

The investigators from this study may want to contact your child in the future regarding this study or to see if she would be interested in participating in future studies. At this time you may decide whether or not you want her to be contacted. If and when she is contacted she may decide if she wants to participate in any other studies and will sign another consent form to participate in those studies. Your decision regarding future contacts will not affect her participation in this study.

Please check one:

- ☐ Yes, she may be contacted in the future regarding this study **or** future studies.
- ☐ Yes, she may be contacted in the future for this study, but **not** for future studies.
- ☐ NO, she may not be contacted in the future.

Blood Samples

If it is okay with you we would also like to collect white blood cells from your daughter's blood so that we can screen for DNA and genes involved in how the body uses nutrients. The samples may be used to help identify genetic factors that influence how the body uses nutrients and to understand how these may be related to differences in bone loss or fetal bone growth. The samples will not be sold or used directly for the production of commercial products and will be kept in a locked lab.

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Reports about future research done with the sample will NOT be kept in your daughter's health records, but the sample reports may be kept with study records or in other secure areas.

You can decide if you want your daughter's sample to be used for this type of research. Your decision can be changed at any time by notifying the study doctor in writing. Your decision about your daughter's sample will not affect her participation in this study or other studies.

Please check one:

- ☐ Yes, you may use my daughter's blood sample for the DNA studies described above.
- ☐ NO, you may not use my daughter's sample for the DNA studies described above.

Signature/Dates

I have read (or have had read to me) the contents of this permission form and have been encouraged to ask questions. I have received answers to my questions. I give my permission for my child to participate in this study. I have received (or will receive) a signed copy of this form for my records and future reference.

Study Subject: _____ Print Name

Parent/Guardian: _____ Print Name and Title

Parent/Guardian: _____ Signature

_____ Date

Person Obtaining Permission

I have read this form to the parent/guardian and/or the parent/guardian has read this form. I will provide the parent/guardian with a signed copy of this form. An explanation of the research was given and questions from the parent/guardian were solicited and answered to their satisfaction. In my judgment, the parent/guardian has demonstrated comprehension of the information.

_____ Print Name and Title

_____ Signature

_____ Date

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Consent Form

Study Title: **Maternal / Fetal Bone Health in Pregnant Adolescents**

Principal Investigators: **Thomas McNanley, M.D., Elizabeth Cooper, CNM, EdD, FACNM**

Co-Investigator: **Kimberly O'Brien, Ph.D.**

Introduction

This consent form describes a research study and what you may expect if you decide to participate. You are encouraged to read this consent form carefully and to ask the person who presents it any further questions you may have before making your decision whether or not to participate.

This study is being conducted by Thomas McNanley, MD, and Beth Cooper, CNM, EdD and Kimberly O'Brien, PhD, of the University of Rochester's Department of Obstetrics and Gynecology and Highland Hospital's Department of Obstetrics and Gynecology. Kimberly O'Brien is also on the faculty at Cornell University.

You are being asked to participate in this study because you are pregnant and 18 years of age or younger.

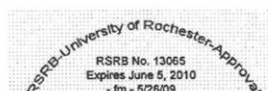
Purpose of Study

The purpose of the study is to learn more about bone health in pregnant teens and about how babies' bones grow before they are born. Calcium and vitamin D are found in foods that you eat and are needed for healthy bones. Teenage girls build most of their bone by the time they are 17-18 years old. When teenagers become pregnant they may need extra calcium. Too little calcium and vitamin D in the diet might cause teenage girls to lose bone. It might also slow the growth of their baby's bones.

You can volunteer for this study if you:

- Are 18 years old or younger
- Are less than 30 weeks pregnant
- Do not have high blood sugar (diabetes)
- Do not have HIV infection
- Do not have problems eating and digesting your food
- Do not have an eating disorder (such as anorexia)

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Description of Study Procedures

If you decide to participate in this study you will be asked some questions to see how healthy you are. We will also look in your medical chart to learn more about how healthy you are and to learn more about your pregnancy. We would like you to come to Highland Hospital up to three times during your pregnancy, with visits usually being spaced at least four weeks apart so we can:

- Measure how tall you are and how much you weigh.
- Take a measurement of your blood pressure and heart rate.
- Measure the distance between your knee and your heel.
- Take a picture of your baby (called an ultrasound or sonogram). This test is safe to use during pregnancy.
- Ask you questions about what you eat and how you decide what to eat, and about how much you exercise.
- Ask you questions about how often you use drugs or alcohol.
- Measure how much bone you have in your body by putting your heel in a special ultrasound machine that will not hurt you or your baby.

Each visit may take up to two hours, so we will offer you some food and a drink during that time.

At one of these visits, when you are approximately **20 - 30 weeks pregnant**, we will also:

- Take a blood sample from your arm (about 4 teaspoons). Before the blood is taken, the nurse will ask you if you want some special cream to be put on your arm to make it hurt less. We will keep this blood sample until the entire sample collected has been used to measure nutrients.

We will use this blood sample to measure:

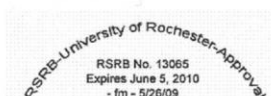
- Hormones and proteins that your body needs when you are pregnant
- Levels of lead in the blood
- Levels of vitamin D in the blood

Lead is found in many homes in Rochester. If it gets into your body it can make you and your baby sick. We will call your doctor right away if you have too much lead in your blood. Your doctor can decide if you need medicine to get rid of this lead. If you have very high lead in your blood, you will not be able to continue in this study.

Second Part of Study (Optional)

If you would like to, you can also participate in the second part of the study on the day your baby is born. It is okay if you do not want to participate in this second study. You can still be in the first part of the study. If you want to participate in this second part, your medical chart will have a sticker put on it. The sticker will let the doctors and nurses know that they should call us after you go into labor and before your baby is born.

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If you decide to participate in part 2, we will:

- Collect a sample of your blood (3 teaspoons). A blood sample is normally taken from your arm when you enter the hospital to have your baby and the sample we are collecting will be drawn at the same time. We will use this sample and the blood sample from the placental cord to measure hormones and proteins that your body needs when you are pregnant.
- Record the birth weight and birth length and other health information on your baby from your baby's medical chart.
- After a baby is born, the baby's afterbirth (called the placenta) comes out. The placenta is usually thrown away as waste. Before it is thrown away we will take some pieces of the placenta. This will help us to learn more about how your placenta sent nutrients to your baby while you were pregnant. We will also take some blood from the cord of the placenta (2-3 tsp.). This cord blood sample will tell us about the nutrients and hormones that your baby has when he/she is born. We will keep the blood samples until the entire sample has been used to measure nutrients.

We will use the blood sample we get from the placental cord blood to measure:

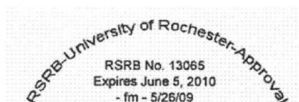
- Hormones and proteins that your baby needs for healthy bones
- Levels of lead in the blood
- Levels of vitamin D in the blood

If you have lead in your body some of this lead may be sent to your baby while you are pregnant. This lead can make your baby sick. If your baby has too much lead in their cord blood, we will call your baby's doctor right away. Your baby's doctor can decide if your baby needs medicine to get rid of this lead.

We would also like to measure how much bone you and your baby have in your bodies using a special test called a DXA. The DXA machine is located at Strong Memorial Hospital. We will try to schedule this test within 4 weeks of your baby's delivery and ask you to come to Strong Memorial Hospital with your baby for this final test.

The Dual energy X-ray absorptiometry (DXA) scan will measure the bone density of your whole body and of your lower spine. For the whole body scan, you will be asked to lie flat on your back on a table as the scanning machine moves above your body. From the scan of your whole body we will be able to see how much calcium you have in your entire body. We can compare this to the amount that is normally found in teenagers that are your age. This test will also measure the amount of fat and lean tissue in your body. For the scan of your lower back you will be asked to also lie on your back on the padded table, a large fabric covered foam cube will be placed under your lower legs so that you are in the best position to take the measure of your lower back area.

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These DXA scans are like an X-ray, and these tests will take about 5-10 minutes to complete. It is safe to use if you are not pregnant. You should not have this test if you may be pregnant. Therefore, you will need to read and sign the "DXA waiver" and you may be tested for pregnancy before the DXA using a urine test.

The DXA test on your newborn will take approximately 2 minutes. Your baby will be wrapped in a blanket and lie on a padded table for the test. You can stay in the room with your baby while the test is conducted. To complete the entire visit for this study should take 40-60 minutes.

Number of Subjects

We plan to recruit a total of 300 adolescents to volunteer for this research study.

Risks of Participation

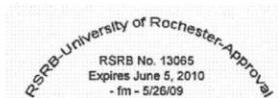
There are no known risks from the ultrasounds that we will take of your heel and of your baby. A total of 4 teaspoons of blood will be taken during pregnancy. A total of 7 teaspoons of blood will be taken if you volunteer for both studies. You may get a bruise and it may hurt a bit when the blood samples are taken. Some people feel lightheaded or faint when their blood is drawn. There is also a rare risk of infection. The samples of the placenta are collected after the baby is born and will not cause any risk. Use of the cream to numb your arm may cause irritation.

The DXA scans you will receive from getting the bone density test if you participate in part 2 of the study(the delivery part) involve radiation exposure which is less than a person would receive from a chest X-ray or a dental X-ray. The radiation amount is about what you would get in one day from the natural radiation around us. The amount of radiation your baby will receive from this test is also similar to what he/she receives from the natural background each day. We don't know whether any radiation dose is completely safe. The risk from the dose you and your baby will receive is considered low compared to other everyday risks.

Benefits of Participation

Screening for lead in your blood during pregnancy may be a benefit. High blood levels can be treated. We will tell you and your primary care provider if we find high lead levels. We will also test your blood for levels of vitamin D. Low vitamin D can be treated with supplements. If you are found to have low vitamin D levels we will tell you and share this information with your primary care provider. There is no health benefit provided by ultrasound screening. The ultrasound of your baby is only to tell us about the growth of your baby. However, if we found anything that made us worry that your baby was not healthy, we would tell you and your obstetrician right away.

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Alternatives to Participation

You do not have to participate in this study if you do not want to. Your decision not to join this study will not affect the health care you receive at Highland Hospital or elsewhere.

Payments

Part 1:

For completing the **first visit** you will be given;

- A sonogram picture of your baby
- A small photo album

For completing the **second visit** you will be given;

- A sonogram picture of your baby
- A \$10 Target or Walmart gift card (you can decide which store you prefer)

For completing the **third visit** you will be given;

- A sonogram picture of your baby
- A \$15 Target or Walmart gift card (you can decide which store you prefer)

Part 2:

If you participate in part 2 when your baby is born we will take a digital picture of you with your baby, if you would like. The picture is a gift and will not be saved or used for other research purposes. You can have your picture taken in the hospital or during your post partum visit at RAMP.

On the day that you come into the GCRC at Strong Memorial Hospital for the DXA study you will receive \$25.00 in cash.

Sponsor Support

The University of Rochester is receiving payment from the USDA for conducting this research study.

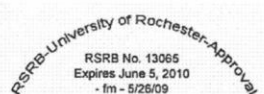
Confidentiality of Records and HIPAA Authorization

While we will make every effort to keep information we learn about you private, this cannot be guaranteed. Other people may need to see the information. While they normally protect the privacy of the information, they may not be required to do so by law. Results of the research may be presented at meetings or in publications, but your name will not be used.

The federal Health Insurance Portability and Accountability Act (HIPAA) requires us to get your permission to use health information about you that we either create or use as part of the research. This permission is called an Authorization. We will use;

- Demographic information (where you live, your phone number, etc.)
- Information on your height, weight and previous pregnancies
- Dietary information and information on supplement use over pregnancy
- Self reported drug and alcohol use and use of cigarettes
- Current use of medications and prescription drugs
- Diagnosis of any pregnancy complications or health problems

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- Test results on hemoglobin and routine tests drawn across pregnancy
- The place where you were seen
- The name of your physician
- The medical records of your newborn

We will use your health information to conduct the study and to determine how your health status and other medical care issues that are happening during your pregnancy might be influencing the health of your bones and the growth of your developing baby. Health information is used to report results of research to sponsors and federal regulators. The health information collected may be audited to make sure we are following regulations, policies and study plans. URM/Strong Health policies let you see and copy health information we have gathered for this research study after the study ends, but not until the study is completed. If you have never received a copy of the URM/ Strong Health HIPAA Notice of Privacy Practices, please ask the investigator for one.

To meet regulations or for reasons related to this research, the study investigator may share a copy of this consent form and records that identify you with the following people. The University of Rochester; the Department of Health and Human Services; the United States Department of Agriculture, Cornell University, University of Rochester, Highland Hospital, and your primary care provider.

If you decide to take part, your Authorization for this study will not expire unless you cancel (revoke) it. The information collected during your participation will be kept indefinitely. You can always cancel this Authorization by writing to the study investigator. If you cancel your Authorization, you will also be removed from the study. However, standard medical care and any other benefits to which you are otherwise entitled will not be affected. Canceling your Authorization only affects uses and sharing of information after the study investigator gets your written request. Information gathered before then may need to be used and given to others.

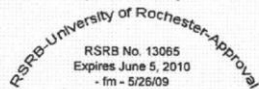
As stated in the section on Voluntary Participation below, you can also refuse to sign this consent/Authorization and not be part of the study. You can also tell us you want to leave the study at any time without canceling the Authorization. By signing this consent form, you give us permission to use and/or share your health information as stated above.

Contact Persons

For more information concerning this research, please contact: Beth Cooper in the RAMP clinic at (585) 275-2962 or Kimberly O'Brien at (607) 255-3743.

If you have any questions about your rights as a research subject, you may contact the Human Subjects Protection Specialist at the University of Rochester Research Subjects Review Board, Box 315, 601 Elmwood Avenue, Rochester, NY 14642-8315, Telephone (585) 276-0005, for long-distance you may call toll-free, (877) 449-4441. You may also

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contact the Cornell University Committee on Human Subjects (UCHS) at 607-255-5138, or via the web at: <http://www.osp.cornell.edu/Compliance/UCHS/homepageUSHS.htm>.

Voluntary Participation

Participation in this study is voluntary. You are free not to participate or to withdraw at any time, for whatever reason, without risking loss of present or future care you would otherwise expect to receive. In the event that you do withdraw from this study, the information you have already provided will be kept in a confidential manner.

Subject Consent

Second Part of Study

Please check one:

- ☐ I also agree to participate in the second part of the study when my baby is born.
- ☐ I do not agree to participate in the second part of the study when my baby is born.

Future Studies

The investigators from this study may want to contact you in the future regarding this study or to see if you and/or your child would be interested in participating in future studies. At this time you may decide whether or not you want to be contacted. If and when you are contacted you may decide if you and/or your child want to participate in any of the other studies and will sign another consent form to participate in those studies. Your decision regarding future contacts will not affect your participation in this study.

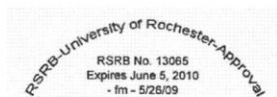
Please check one:

- ☐ Yes, I may be contacted in the future regarding this study **or** future studies.
- ☐ Yes, I may be contacted in the future for this study, but **not** for future studies.
- ☐ NO, I may not be contacted in the future.

Blood Samples

If it is okay with you we would also like to collect white blood cells from your blood so that we can screen for DNA and genes involved in how the body uses nutrients. The samples may be used to help identify genetic factors that influence how the body uses nutrients and to understand how these may be related to differences in bone loss or fetal bone growth. The samples will not be sold or used directly for the production of commercial products and will be kept in a locked lab. Reports about future research

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done with the sample will NOT be kept in your health records, but the sample reports may be kept with study records or in other secure areas.

You can decide if you want your sample to be used for this type of research. Your decision can be changed at any time by notifying the study doctor in writing. Your decision about your sample will not affect your participation in this study or other studies.

Please check one:

- ☐ Yes, you may use my blood sample for the DNA studies described above.
- ☐ NO, you may not use my sample for the DNA studies described above.

Delivery Calcium Study

If you are interested, you can also participate in a smaller sub-study of the Bone Health study. This study is designed to learn how the calcium that you eat crosses the placenta to your baby. This smaller study would take place at Highland hospital when you go into labor to deliver your baby. It would involve one more blood sample from you, for which you would receive an additional \$25 in cash.

Please check one:

- ☐ Yes, I am interested in hearing more about the Delivery Calcium study.
- ☐ NO, I am not interested in hearing about the Delivery Calcium study.

Signature/Dates

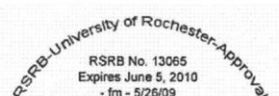
I have read (or have had read to me) the contents of this consent form and have been encouraged to ask questions. I have received answers to my questions. I agree to participate in this study. I have received a signed copy of this form for my records and future reference.

Study Subject: _____ Print Name

Study Subject: _____ Signature

_____ Date

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Person Obtaining Consent

I have read this form to the subject and/or the parent/guardian has read this form. I will provide the subject and parent/guardian (if present) with a signed copy of this consent form. An explanation of the research was given and questions from the subject were solicited and answered to the subjects satisfaction. In my judgment, the subject has demonstrated comprehension of the information.

Print Name and Title

Signature

Date